THE JOURNAL OF GENERAL PHYSIOLOGY

Founded by Jacques Loeb.

EDITORS

W. J. CROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

VOLUME SEVENTH

WITH 5 PLATES AND 190 FIGURES IN THE TEXT

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PAGE

MEMBRANE EQUILIBRIA AND THE ELECTRIC CHARGE OF RED BLOOD CELLS.

By CALVIN B. COULTER.*

(*From the Pathological Laboratory, Brooklyn Hospital, and the Department of Bacteriology, Columbia University.*)

(Accepted for publication, May 6, 1924.)

The present work is an investigation into the electric charge of

CORRECTION.

On page 649, Vol. vi, No. 6, July 20, 1924, in Table I, in the heading for the fourth column, for *Relative critical energy, lamp and filter* read *Relative energy, lamp and filter.*

In the same column, the last figure, for *381.0* read *38.1.*

have applied this theory to the red cell plasma system in papers which have appeared while the present work was in progress, and which deal with ion concentrations that apply to the physiological rôle of the

*Van Cott Fellow in Pathology.

[1] Höber, R., *Arch. ges. Physiol.*, 1904, ci, 607.

[2] Ostwald, W., *Z. physik. Chem.*, 1890, vi, 71.

[3] An interpretation of the theories of Donnan is given by Lewis, W. C. McC., A system of physical chemistry, London and New York, 3rd edition, 1920, ii, and by Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922.

[4] Warburg, E. J., *Biochem. J.*, 1922, xvi, 153.

[5] Van Slyke, D. D., Wu, H., and McLean, F. C., *J. Biol. Chem.*, 1923, lvi, 765.

cells. The numerous papers of Jacques Loeb appearing in the *Journal of General Physiology* on the application of the Donnan equilibrium to protein systems present the background of the present study.

Observations reported in a previous paper[6] had shown difference between the pH of the interior of red cells and that of the medium, so large that it seemed impossible to explain them by the Donnan equilibrium. It was found further that fresh red cells, when washed and suspended in saccharose solution showed a higher charge in cataphoresis than similarly washed cells which had been preserved 24 hours or longer in saccharose solution. This made it appear doubtful that the ionization of the cell protein was the sole source of the charge and it seemed possible that the cells owed their charge in part at least to a simple diffusion potential, due to diffusion outward of salt contained within the cell. Such a charge might be high when the cells were first placed in an electrolyte-free medium, and would fall to zero as soon as diffusion had brought the salts to the same concentration on both sides of the cell membrane. In view of the observed changes in pH of the medium, the most important salt in this connection appeared to be the phosphates of the cell. Accordingly the amount of phosphate which had diffused out of the cells was determined by the method of Tisdall[7] in the supernatant fluid from equal amounts (4 cc.) of washed sheep cells which had been allowed to stand for varying lengths of time in like quantities (40 cc.) of isotonic saccharose solution at about 10°C.

One experiment, which was repeated numerous times with the same general result, is given in Table I.

The amounts are so small that their exact determination is at the limit of accuracy of the method, but the relative error between determinations is probably small. It is apparent that the greater part of the total PO_4 which diffuses from the cells in 24 hours does so within the first 5 minutes or less. The subsequent rate of diffusion is so low that the condition approximates an equilibrium in the distribution of PO_4 ions between the cell and the medium. A potential due to diffusion of molecular phosphate must therefore be infinitesimal.

[6] Coulter, C. B., *J. Gen. Physiol.*, 1921–22, iv, 403.

[7] Tisdall, F. F., *J. Biol. Chem.*, 1922, l, 329.

Measurements of the H concentration in the medium and in the interior of cells give evidence of the attainment of a like condition of equilibrium in the distribution of this ion. This is true both when the medium is originally electrolyte-free and when it contains salts, although the H distribution is markedly affected by the presence of salts.

Cells from fresh defibrinated sheep blood were washed twice with five volumes of isotonic NaCl solution and four times with similar volumes of saccharose solution; 4 cc. of strongly sedimented cells were added in the majority of experiments to 36 cc. of isotonic solution, in stoppered tubes. These were placed in the refrigerator at 5°–15°C. After varying intervals the supernatant fluid was separated by the centrifuge and removed as completely as possible without disturbing the cell sediment. The latter was then dissolved in 20 cc. of distilled water. The water used for all solutions was distilled, with occasional exceptions, the same day it was used. The pH measurements were made with freshly palladinized electrodes in vessels similar to that of Clark,[8] using a separate electrode and vessel for each specimen of fluid. The vessels were not agitated. The battery of electrodes was placed in the refrigerator for 12 to 18 hours, removed one at a time, hydrogen admitted to restore atmospheric pressure, and the E.M.F. read 10 minutes after removal from the refrigerator. The temperature of the solution was taken immediately after the E.M.F. was read. The electrodes were allowed barely to touch the surface of the solutions; those containing dissolved cells showed, after the prolonged stay in the refrigerator, a narrow zone, at the surface, of completely reduced hemoglobin. The usual technique was em-

TABLE I.

Mg. × 10^{-3} Phosphorus Lost by 4 Cc. of Cells.

5 min.	1 hr.	3 hrs.	24 hrs.
9.0	9.5	10.4	11.2

[8] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

ployed by changing the solution to maintain approximately the original concentration of CO_2 of the solution. Measurements by this method were more consistent than those in which electrode equilibrium was sought by rocking the vessel; the solutions containing much hemoglobin gave considerably higher pH values which could be reproduced within 0.01 pH. Control measurements in cell solution diluted 1:10, 1:5, and 1:1, the last made by freezing and thawing the cell sediment, gave in one experiment the following values: 1:10, pH 7.277; 1:5, pH 7.273; 1:1, pH 7.253. In several other experiments the 1:10 dilution gave values from 0.03 to 0.06 pH higher than the

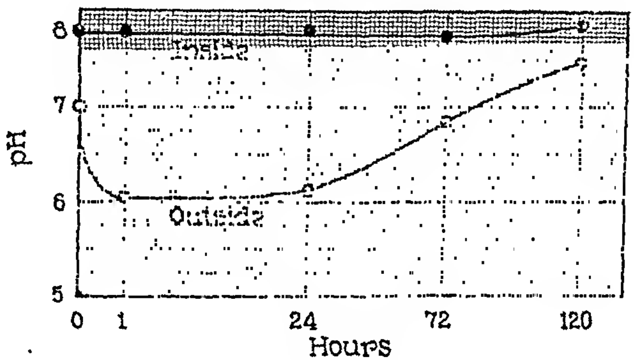

FIG. 1. Reactions of medium and cells suspended in 250 cc. of saccharose solution without salt.

undiluted cell fluid. The change in pH caused by reduction of the oxyhemoglobin to reduced hemoglobin by the hydrogen electrode has been considered too small to require correction for the purpose of this investigation. Frequent colorimetric controls were made of the pH of the outside fluids. Regardless of the method or technique employed for pH measurement, the relations between inside and outside pH were always of the same nature as those shown in the curves.

The buffer value of the cell interior shown by the figures for the effect of dilution in pH is thus very high, and we feel justified in concluding that the pH of the cell has not been altered to a significant degree by hydrolysis, on dissolving the cells in water. The pH of the 1:5 cell solution has been assumed to represent the actual pH within the cell.

In Fig. 1 are given the pH values of the inside (or 20 per cent) cell solution and of the outside solution (or suspending fluid) in an experiment in which 4 cc. of cells were suspended in 250 cc. of pure saccharose solution. The large volume of solution was used to keep the concentration of electrolyte, resulting from simple diffusion from the cells, as low as possible. Under these conditions the reaction which is reached in the outside fluid within the first hour persists without significant change for 24 hours. Other experiments show that this reaction is reached within a few minutes after adding the cells to the solution.

Careful attention has been paid to the apparent increase in acidity of the outside fluid, which reached in the majority of cases about pH 6, but in numerous experiments became as acid as pH 5.6 to 5.8. Hydrogen electrode measurements in solutions of so low conductivity and buffer value as these are difficult and uncertain; nevertheless saccharose solutions made with freshly distilled water gave repeatedly pH values of 6.8 to 7.0, and there could be no doubt of an actual increase in acidity of the solution after contact with the cells. It was given by both electrometric and colorimetric methods, and has been confirmed by Eggerth.[9] It was found that it was not due to a diffusion of CO_2 from the cells. Since it represents a movement of H from a region of low concentration (the cell interior) to one of high, with respect to this ion, it is evident that the H ion, in spite of its great mobility, is not primarily concerned in the process.

The gradual increase in alkalinity of the outside fluid which is observed after 24 hours is due probably to an alteration in the permeability of the cell membrane. If the volume of solution in which the cells are suspended is small, 40 cc. instead of 250 cc., the outside fluid shows the initial increase in acidity described, which is often sufficient to produce immediate agglutination of the cells, and undergoes after 2 to 4 hours a steady rise in alkalinity, reaching as a rule about pH 6.8. With this change in reaction, the stability of the cells increases, and they remain uniformly suspended after gentle agitation of the vessel containing them.

The inside reaction undergoes very slight change, if the cell suspension be kept at 5°C. However, if the cells are stored at room

[9] Eggerth, A. H., personal communication.

temperature, a distinct rise in alkalinity may be observed after a few hours. It seems possible that this is due to the formation of NH_3 within the cell.

If cells which have been washed in saccharose solution be suspended in isotonic solutions of NaCl or $CaCl_2$ the increase in acidity of the outside fluid does not occur, but the pH of this fluid rises and that of the inside falls within a few minutes, so that they approximate each

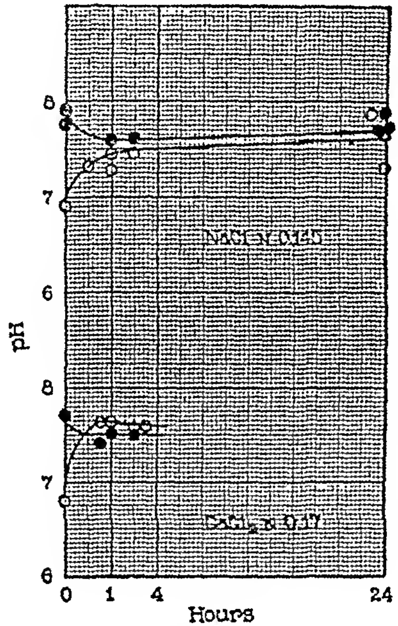

FIG. 2. Reactions of medium and cells suspended in isotonic NaCl and $CaCl_2$ solutions. Black circles, inside pH; white circles, outside pH.

other. This is shown in Fig. 2, which represents two experiments with each of the two salts. In the majority of experiments with NaCl the outside reaction remained slightly more acid than the inside, although in several experiments at the end of 24 hours in the refrigerator the two were identical. With $CaCl_2$ the outside pH rises higher and the inside pH falls lower than in the case of NaCl, so that the inside reaction is now the more acid. Experiments which for the sake of brevity are not detailed have shown that if the concentration of the salt in the suspending fluid is less than isotonic, the isotonicity

of the solution being maintained by saccharose, the changes in pH a intermediate between those just described and those that occur wh the outside fluid contains no salt. The extent of change increas with the concentration of the salt.

The reaction attained in the outside fluid when acid is added affected in a similar way by the presence of salts. In the experime recorded in Fig. 3, the salts were present in one-quarter of their isoto concentration, and the same amount of HCl was added before t addition of cells to each of the solutions, bringing them approximat to pH 3.3. The outside reaction remains more acid in the salt-f

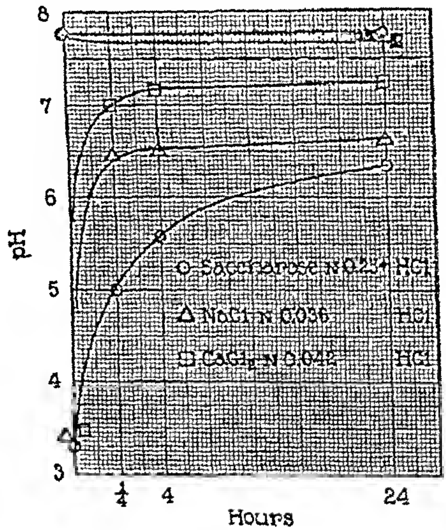

FIG. 3. Reactions of cells and medium to which HCl had been added brin it to pH 3.3. The salts were present in one-quarter isotonic concentration. U curves (black circles) inside pH; lower curves (white circles) outside pH.

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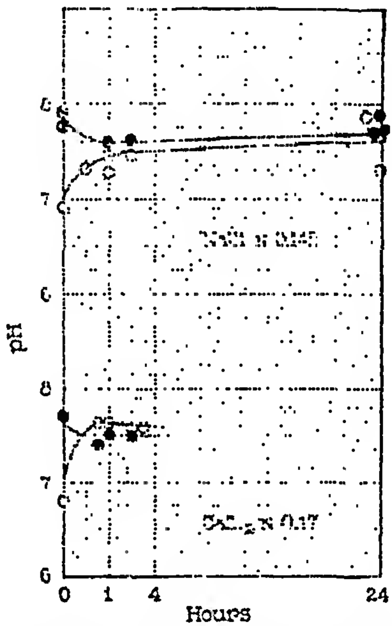

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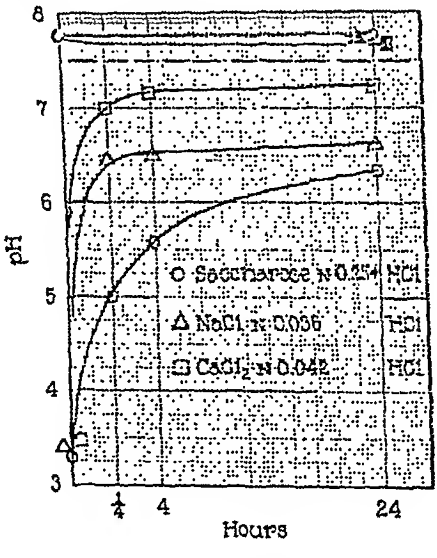

Fig. 3. Reactions of cells and medium to which HCl had been added bringing it to pH 3.3. The salts were present in one-quarter isotonic concentration. Upper curves (black circles) inside pH; lower curves (white circles) outside pH.

solution, and more acid in NaCl than in $CaCl_2$ solution. A close approximation to equilibrium between 4 and 24 hours is evident in the case of the salt solutions. The result is similar when the acid present is H_2CO_3 instead of HCl. Solutions of saccharose and of NaCl, KCl, and $CaCl_2$ were saturated with alveolar air, at about 15° C., bringing them all to the same pH (colorimetric) in each experiment. 4 cc. of packed cells were added to each of the solutions, which

had been covered with a layer of paraffin oil. The concentrations of the salts and the reactions after 80 minutes at 10°C., in two experiments are given in Table II.

The effect of salts is thus the same as when HCl is added. In the more concentrated $CaCl_2$ solution the original relation is reversed and the inside reaction becomes definitely more acid than the outside, as is the case when no acid is added, shown in Fig. 2. Determinations of combined CO_2 in the cells and in the outside fluid gave values which were so irregular as to be without significance.

In order to determine more exactly the distribution of H and Cl ions on the two sides of the cell membrane at equilibrium a separate series of measurements were made with cells which were washed once

TABLE II.

Effect of CO_2 on Reactions of Cells and Medium.

Suspending fluid.	pH outside.	pH inside.
Saccharose	6.27	7.54
KCl N 0.018	6.91	7.23
NaCl N 0.018	6.94	7.27
$CaCl_2$ N 0.021	7.11	7.08
Saccharose	5.65	7.78
NaCl N 0.145	7.43	7.40
$CaCl_2$ N 0.17	7.46	7.31

with saline solution and twice only with saccharose solution, and which were allowed to remain in contact with the outside fluid for 2 hours in the refrigerator. The Cl ratios were determined with AgCl electrodes, using a saturated solution of KCl as a reference solution. Electrodes of C.P. silver were coated with AgCl by electrolysis in an acid solution of N 0.145 NaCl and after thorough washing were allowed to remain in distilled water in the dark for 18 to 48 hours. The electrodes were placed in vessels such as were used for pH measurement. These are provided with a 4-way stop-cock at the lower end so that the two vessels may be connected with a short length of rubber tubing and a salt bridge established by connection with a reservoir of saturated KCl. The contact of the solution to be measured with the salt bridge was made by turning the 4-way cock, the bore of which

contained saturated KCl, until the periphery of this bore was separated from the vessel opening containing the unknown solution by about 2 mm., the stop-cock being greased, and the potential difference between the two electrodes measured with a potentiometer and a sensitive high resistance galvanometer. It was necessary to make the reading very rapidly, before the saturated KCl had opportunity to diffuse into the unknown less concentrated solution. All measurements were made in triplicate, with close agreement between the highest readings on each solution. In order to calculate the pCl of the outside solutions from these E.M.F. values, the E of the saturated KCl electrode was determined by measuring the E.M.F. of the cell: saturated KCl – N 0.1 KCl, and assuming an activity of 0.08 for the N 0.1 KCl solution. From this was obtained the value 0.0286 as the E.M.F. of the saturated KCl electrode against a solution normal with respect to Cl ion. The calculation of pCl from the E.M.F. between the unknown solution and the saturated KCl solution is then at 22° C.

$$\frac{\text{E.M.F. observed} - 0.0286}{58.5} = \text{pCl}$$

The packed cell sediment was suspended in saccharose solution to give a 40 per cent suspension; of this 10 cc. were added to 30 cc. of various concentrations of NaCl and $CaCl_2$ in saccharose solution. After 2 hours stay in the refrigerator, with occasional gentle agitation of the vessels, the supernatant fluid was separated completely after centrifugation and the cells dissolved by adding distilled water to 40 cc., giving a 10 per cent solution. The measurements with H_2 and AgCl electrodes were made on this dilute solution and on the supernatant fluid.

These measurements give the thermodynamic P.D. in millivolts between the two sides of the cell membrane, which is due to the difference in concentration of H or Cl, by subtracting the E.M.F. given by the more concentrated solution, with respect to H or Cl, from the E.M.F. given by the more dilute solution, when measured against a saturated calomel electrode in the case of the H electrode or against the saturated KCl electrode in the case of the Cl electrode measurements. In the latter case the value 58.5 must be subtracted from the

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E.M.F. of the cell solution to correct for the 1:10 dilution of the cells. The calculation is as follows:

Pd H_2 — Saturated calomel chain: E.M.F. inside — E.M.F. outside = H P.D.
AgCl — Saturated KCl chain: E.M.F. outside — (E.M.F. inside — 58.5) = Cl P.D.

The values of H P.D. and Cl P.D. have been plotted as ordinates against the pCl of the outside fluid as abscissæ and are shown as experimental points along the two upper curves in Fig. 4.

If the Donnan equilibrium holds for the distribution of ions between the cell and the medium we should expect these two sets of values to be identical. Experimentally the Cl P.D. is consistently lower than the H P.D. It has been assumed that cells strongly sedimented in the centrifuge are actually concentrated; this was not the case since the cell mass did not show the transparency which develops when intervening fluid is entirely displaced from between the cells. Consequently the actual volume of the cells was less than 4 cc., and the actual Cl P.D. should be greater than that observed. The determinations of the H electrode E.M.F. of the cell solutions may be affected with a considerable experimental error, in spite of the reproducibility of results and are apparently too high. In hydrogen electrode measurements of hemoglobin solutions one is confronted with two sources of error: incomplete reduction, leading to low E.M.F., or the occurrence of chemical changes in the solution, leading possibly to an actual increase in alkalinity. It seemed most important to avoid incomplete reduction and the technique described was devised. It offers the possibility that the unexpectedly high cell pH values are due to the formation of NH_4. However, if H_2 be passed through a fresh hemoglobin solution continuously to a point of complete reduction, NH_4 formation must be insignificant, and the pH approximates but is slightly higher than that given by the technique of prolonged stay in a resting H_2 atmosphere. Furthermore the hemoglobin existed in the cells as oxyhemoglobin but was measured as reduced Hb; correction of the E.M.F. for the increase in alkalinity resulting from reduction would tend to make the H and Cl electrode values approximate, more closely than shown in Fig. 4.

Since the H is concentrated on that side of the cell membrane (outside) on which the Cl is dilute, in all the experiments of Fig. 4,

the P.D. has the same sign of charge in both H and Cl electrode measurements, and in either case the electrode placed in the outside fluid is positive in sign against the electrode which we imagine to be placed within the cell. Further, since the P.D. is determined by the ratio between the concentration inside the cell and that in the outside fluid,

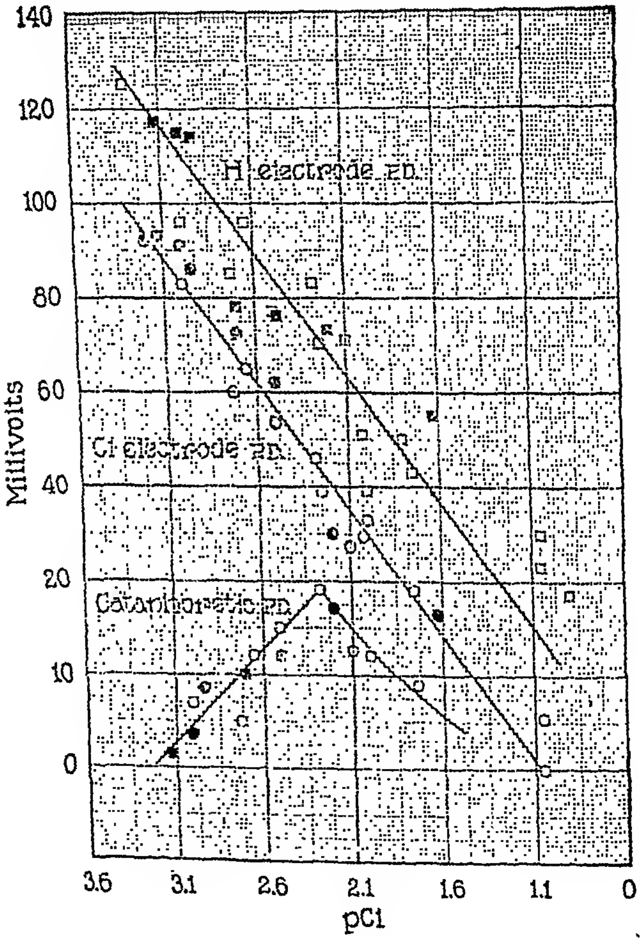

FIG. 4. H_2 electrode P.D., upper curve, the points represented by squares; Cl electrode P.D. middle curve, the points represented by circles. Cataphoretic P.D lower curve. Black squares and circles, $CaCl_2$; white squares and circles, NaCl. The ordinates give P.D. in millivolts, the abscissæ measured pCl values of the outside fluid.

the observed values satisfy the equation $\frac{C_{H_1}}{C_{H_2}} = \frac{C_{Cl_2}}{C_{Cl_1}}$ which is required by the Donnan equilibrium, within the limit of accuracy of the technique.

The effect of the electrolyte upon the reactions of the cell and the medium shown in Figs. 1, 2, and 3 is thus a necessary consequence of the Donnan equilibrium. The points for NaCl in Fig. 4 fall as close to each curve as those for $CaCl_2$, so that the effect of the Ca appears to be negligible as showing any differences from Na. In the curves of Figs. 1, 2, and 3 the greater effect of $CaCl_2$ in causing changes in reaction is probably due, therefore, to the greater concentration of Cl in solutions of this salt than in the approximately equimolar solutions of NaCl.

The P.D. arising from differences in concentration of H and Cl on opposite sides of the cell membrane may be compared in Fig. 4 with the P.D. of the cell determined by cataphoresis. The measurements were made with the macroscopic apparatus and technique described in a previous paper [10] at the same time and on the same specimens of cells which were used for the H and Cl electrode determinations. The calculation of P.D. from the observed rate of movement was made from the Helmholtz-Lamb equation, as used by Northrop [11] assuming a relative viscosity of 1.1 for the isotonic saccharose solution compared with pure water. Criticism has recently been made of such evaluation of cataphoresis data by Winslow[12] and his coauthors. The P.D. values so calculated are, however, as nearly accurate as seems at present possible and may be compared with other P.D. values similarly calculated.

The cataphoretic P.D. of the cells is shown in the lower curve of Fig. 4. The P.D. is of the opposite sign, and is first increased by the same low concentrations of Cl which decrease the H and Cl electrode potential. Such a charging effect low of concentrations of NaCl and $CaCl_2$ is evident in the experimental points given by Loeb, in cata-

[10] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

[11] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

[12] Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F., *J. Gen. Physiol.*, 1923–24, vi, 177.

phoresis of gelatin-coated and casein particles[13] at reactions on the alkaline side of the isoelectric point, and is very prominent in the cataphoresis of collodion particles.[14] After a maximum is reached, the cataphoretic P.D. of red cells falls with increasing Cl concentration, as does the H and Cl electrode potential, but remains consistently lower.

The marked valency effect of Ca in higher concentrations of $CaCl_2$ than those of Fig. 3 and of La in very low concentration upon the

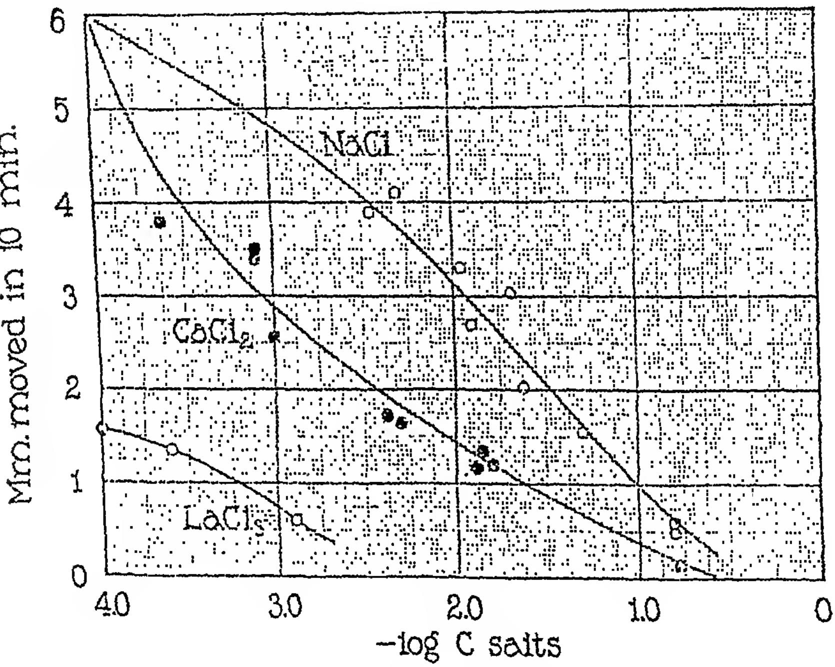

FIG. 5. The rate of movement of red cells under a potential gradient of 3.9 volts per cm. in the presence of NaCl, $CaCl_2$, and $LaCl_3$, at pH 7.

cataphoretic P.D. is shown in Fig. 5, in which the actual rates of movement in mm. per 10 minutes are plotted against the negative log of the molar concentration of the salts calculated from their dilution. The rates shown were extrapolated on a curve drawn between 0 at pH 4.7[10] and the value observed at the pH of the suspending fluid, in

[13] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.
[14] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109.

order to give the rate of movement at pH 7. The curve for extrapolation was drawn conformable to that for the migration of cells in the absence of added salt. The error involved is not of significance as the actual pH values fell between pH 7.1 and 6.2. Only the discharging effect of the salts is shown in Fig. 5 since the left-hand extremity of the curve represents the maximal rate of movement observed in previous experiments at pH 7. The data for this curve were obtained before the charging effects of low concentrations of salts had been observed, and the pCl of the medium was not measured.

It is evident that the cataphoretic P.D. of red cells is lower than the thermodynamic P.D., and is affected differently by the presence of salts. The valency influence of the cation of the salt is not apparent in the charging effect of low concentrations of NaCl and $CaCl_2$, although the experimental inaccuracies may render this point somewhat uncertain; the valency influence is well marked with high concentrations of NaCl and $CaCl_2$ and with $LaCl_3$. This is the usual effect. No influence of the cation is observed in the reduction of the thermodynamic potential; the effect of salts is shown only in the concentration of the Cl. Differences between the two types of potential appear to prevail generally; they were observed by Loeb in extensive observations on various types of suspended particles.[15,16]

DISCUSSION.

It might be supposed that it is the impermeability of the cell membrane to the protein ions within the cell which would be responsible for a Donnan equilibrium between the cell and the medium. That is apparently not the cause of the unequal distribution of H and Cl reported here. At the cell reactions observed, the hemoglobin must exist as K Hb and Na Hb, and if the protein ions were responsible for the Donnan equilibrium, we should expect a membrane hydrolysis to occur, with the development of a more acid reaction within the cell and a more alkaline reaction in the medium. Exactly the opposite change occurs, and we note further that the cation of salts present in the suspending medium has no apparent effect on the

[15] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 505.
[16] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 307.

thermodynamic P.D. We are led, therefore, to the conclusion that it is the cations, K and especially Na in the case of sheep blood cells, which are responsible for the equilibrium. Gürber[17] and Doisy and Eaton[18] have shown by direct analyses that K and Na do not pass through the cell membrane, and Van Slyke[5] and his collaborators have considered this fact fundamental in interpreting the electrolyte and water distribution in the blood on the basis of the Donnan equilibrium. The concentration of these cations within the cell is considerably greater than that of protein ions, and Michaelis[3] has considered the probability of potential differences across the cell membrane due to impermeability to inorganic cations. The mechanism of the observed changes in reaction appears to be as follows: in a salt-free medium, the permeating Cl tends to diffuse from the cell because of the great difference in concentration on the two sides of the cell membrane, and since K and Na do not permeate, the Cl carries with it H until the pressure of this ion on the outer side of the membrane equals that of the Cl from the inside. The interior reaction of the cell does not change appreciably because of the high buffer value of the cell.

It is suggested that this is the mechanism of the production of HCl by the cells of the gastric mucosa. To produce the high acidity of the gastric secretion by this mechanism would require the maintenance of a very high Cl concentration within the cell, as well as a means for the removal of the excess of OH ion within the cells.

Increasing concentration of Cl in the outside fluid may be regarded as opposing the diffusion pressure of Cl from the inside, and if the outside C_{Cl} is the greater, Cl will enter the cell taking with it H and increasing the acidity of the cell. This is the result observed with isotonic NaCl and $CaCl_2$ solutions in Fig. 2 and in Table II. If Fig. 2 be compared with Fig. 3, it is seen that a solution of low C_H but high C_{Cl} produces a greater increase in acidity within the cell than one of of high C_H but low C_{Cl}. It is well known that the presence of neutral salt makes possible the appreciation of sour taste in a solution of less acidity than in one in which neutral salt is absent.

[17] Gürber, A., *Jahres.-ber. fortschr. Thier-chem.*, 1895, xxv, 165. Quoted by Van Slyke.[5]

[18] Doisy, E. A., and Eaton, E. P., *J. Biol. Chem.*, 1921, xlvii, 377.

If the HPO_4'' ions readily permeate the cell membrane we should expect the ratio $\sqrt{\frac{C_{HPO_4''}\text{ inside}}{C_{HPO_4''}\text{ outside}}}$ at equilibrium to equal $\frac{C_{Cl}\text{ inside}}{C_{Cl}\text{ outside}}$. Although we have found by analyses that the total amount of inorganic phosphate lost by the cell to the medium increases slightly with increasing salt concentration of the medium, the ratio of $\frac{\text{inorganic phosphate inside}}{\text{inorganic phosphate outside}}$ is not that to be expected, since in several experiments it was approximately $\frac{100}{1}$ in cases where the Cl ratio $\frac{\text{inside}}{\text{outside}}$ was very nearly 1. It seems probable, therefore, that the distribution of HPO_4'' is not determined by the same mechanism as that of Cl, and a relative impermeability of the cell membrane to HPO_4'' under the conditions of these experiments, is suggested. The determination of HPO_4'' concentration within the cell is subject to far greater difficulty than is that of Cl. The discrepancy observed may also be due to the fact that only a small proportion of the total phosphate within the cell is in the form of ions.

The impermeability of the cell membrane to inorganic cations is not absolute, since resistant specimens of cells may show after 3 to 5 days suspension in a large volume of saccharose solution such a loss of their electrolyte content, without losing hemoglobin, that they do not undergo hemolysis in distilled water until fifteen times their volume of water is added. When examined microscopically in suspension in five times their volume of distilled water such cells appear wholly normal. The reactions of cell and medium at such a time are those shown in Fig. 1. On the other hand, in solutions of relatively high salt concentration the membrane shows a considerable permeability to hemoglobin, although the Donnan equilibrium applies in this case almost as well as where the membrane is entirely impermeable to hemoglobin.

In the case of gelatin particles studied by Loeb,[19] at acid reactions, at which the non-permeating ion is the gelatin cation, the cataphoretic

[19] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 215.

charge is positive in sign, and we should expect that result in the case of red cells, since the non-permeating cations appear to determine the Donnan equilibrium. With both red cells, at the H and Cl concentrations considered here, and acid gelatin particles, the observed thermodynamic potential would be negative in sign if one H (or Cl) electrode were placed within the particle, and one H (or Cl) electrode in the fluid medium; the true membrane potential in this case should be positive in sign, since it is this potential which maintains equilibrium by opposing the thermodynamic or diffusion-pressure potential (Michaelis[3]). This sign of charge corresponds with the observed cataphoretic charge of acid gelatin particles. The cataphoretic charge of red cells in all the experiments of Figs. 4 and 5 is beyond question negative in sign. We should expect it to be positive if both thermodynamic and cataphoretic P.D. have the same origin, as suggested by Wilson.[20] Loeb has considered the valency effect of discharging ions to be strong evidence that the cataphoretic P.D. is determined by the Donnan equilibrium;[16] if this is the case with red blood cells, the cataphoretic P.D. arises conceivably at a different membrane or phase-boundary from that concerned in the thermodynamic P.D. The boundary at which the cataphoretic P.D. is produced in such a case is probably that between the outside of the cell membrane and the medium, since the ionization of the protein of the membrane would be expected to manifest itself here, as it undoubtedly does when the cell assumes a positive charge in reactions more acid than pH 4.7.[10]

SUMMARY.

It has been shown, within the probable limit of error of the methods of measurement employed, that the Donnan equilibrium determines the distribution of H and Cl ions between the cell and the surrounding fluid. This equilibrium is a consequence of the impermeability of the cell membrane to the inorganic cations of the cell. The mechanism responsible for this equilibrium is suggested as that concerned in the secretion of HCl by the cells of the gastric mucosa. If the salt concentration of the medium is low there may result from the Donnan equilibrium a thermodynamic P.D. of considerable magnitude. In

[20] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

the presence of low concentrations of electrolytes, this P.D. is to be regarded as positive in sign at reactions of the medium at which the cataphoretic charge of the cell is negative in sign. The explanation of this discrepancy in sign of charge may lie in the existence at an outer phase-boundary of a second Donnan equilibrium the nature of which is determined by the ionization of the protein of the cell membrane.

SOME PHYSIOLOGICAL ACTIONS OF CYANIDES.

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That cyanides in dilute solutions act as general protoplasmic depressants is well known. In stronger solutions their toxic effects are equally established (Hyman).[1] Inasmuch as these conclusions have been drawn almost exclusively from studies using potassium cyanide, it was thought desirable to see to what extent such effects were due to the alkalinity of the solutions used as well as to their cyanide content.

Potassium cyanide in aqueous solutions is strongly alkaline in reaction due to the manner in which it dissociates.

$$\begin{array}{l} KCN + H_2O \rightleftarrows KOH + HCN \\ \qquad\qquad\qquad\quad \uparrow\downarrow \\ \qquad\qquad\qquad\; K^{\cdot} + OH' \end{array}$$

Hydrocyanic acid, as is well known, is an extremely weak acid, dissociating only to a very slight extent. It is a matter of some importance, therefore, to determine to what extent the physiological properties of a solution of KCN are due to the hydroxyl ions in solution and to what extent they are due to the poorly dissociated HCN molecule. In view of the recent data of Jacobs[2] for carbonic acid and of Beerman[3] for hydrogen sulfide, in which the undissociated molecule has been shown to be of much importance in determining the physiological properties of solutions of these acids, it was thought desirable to study hydrocyanic acid from the same point of view. The experiments herein reported have been carried out with this end in view using various materials: protozoans, tadpoles, and "artificial cells."

[1] Hyman, L. H., *Am. J. Physiol.*, 1919, xlviii, 340.

[2] Jacobs, M. H., *J. Exp. Zool.*, 1912, xii, 519; *Carnegie Institution of Washington, Pub. No. 183*, 1914; *Am. J. Physiol.*, 1920, li, 321; 1920, liii, 457; *J. Gen. Physiol.*, 1922–23, v, 181.

[3] Beerman, H., *J. Exp. Zool.*, 1924, xxxix, (in press).

Experiments on Protozoa.

The effects of CO_2 and of H_2S on various protozoa have been studied by Jacobs[2] and Beerman.[3] These authors found that the order of resistance of various protozoa to these chemicals bears no relation to the order found in the case of other acids which act primarily through their hydrogen ion effects (Collett).[4] Hydrocyanic acid has been found by the author to produce approximately the same effects as carbonic acid and hydrogen sulfide, but with marked differences in velocity for the given species.

Among the forms studied in the present experiments the most extensively used were *Paramecium caudatum*, *Paramecium multimicronucleatum*, *Paramecium aurelia*, *Paramecium bursaria*, *Euplotes patella*, and *Euglena viridis*. Mixed cultures as well as some pure line cultures of these organisms were used. The same calibrated pipette was used throughout, so that the same relative amount of culture was introduced in all experiments. Animals were put in 10 cc. of solutions in Syracuse watch-glasses and kept covered to prevent evaporation. The time taken to kill approximately two-thirds and the time when all were killed was obtained by means of a stop-watch.

Inasmuch as many experiments have been carried out using different concentrations of HCN, and since the results are qualitatively similar, only typical experiments will be cited to show the physiological action of the reagent.

The behavior of protozoa in HCN is strikingly similar to that described by Jacobs[2] and Beerman[3] for CO_2 and H_2S. The animal becomes sluggish, locomotion finally ceases, while the cilia continue beating, eventually stop, and the animal is dead. The bodies of the organisms become slightly swollen, more so in breadth than in length, the nuclei both micro and macro are distinct, while vacuoles from the very beginning of the experiment are engorged and stand out very prominently. No marked cytolysis occurs, except that in *Paramecium bursaria* which is least resistant to the acid the pellicle bursts and an irregularly shaped mass remains after the animal is killed.

[4] Collett, M. E., *J. Exp. Zool.*, 1919, xxix, 443.

In the case of the species of *Paramecia* used the relative toxicity of a solution of HCN of pH 5.6 is as follows: *Paramecium bursaria* is killed in approximately 2 to 3 minutes; *Paramecium aurelia* in 4 to 5 minutes; *Paramecium multimicronucleatum* in 10 to 15 minutes and *Paramecium caudatum* in approximately 1 to 2 hours. *Euplotes patella* is extremely sensitive to HCN, its resistance to the above solution being between that of *Paramecium bursaria* and *Paramecium aurelia*. The time for death ranges from 2 to 3 minutes. *Euglena viridis* is most sensitive to the acid being killed almost instantaneously.

These experiments on protozoa show that different species of these animals have different resistances to HCN. If the effects of this acid are due to hydrogen ions in its solution the same order of resistance ought to be obtained with solutions of other acids, especially when of the same hydrogen ion concentration. The results of Miss Collett[4] and of Jacobs[2] for mineral acids, which have been confirmed by the author, show that the order of resistance of *Paramecia* is *Paramecium bursaria* > *Paramecium caudatum* > *Paramecium aurelia*. The order of resistance to CO_2 (Jacobs[2]) and to H_2S (Beerman[3]) is *Paramecium caudatum* > *Paramecium aurelia* > *Paramecium bursaria*. In the present experiments the order of resistance to HCN is *Paramecium caudatum* > *Paramecium multimicronucleatum* > *Paramecium aurelia* > *Paramecium bursaria*, a condition quite the reverse of that found for mineral acids but similar to that found for CO_2 and H_2S. *Euplotes* is also quite resistant to mineral acids but extremely sensitive to HCN.

Artificial Cell Experiments.

Since Jacobs[2] and Beerman[3] have pointed out that CO_2 and H_2S, in contrast to mineral acids, must act after penetration of the cell, it was thought desirable to test further the possibility that HCN acted in a similar manner. Artificial cells, "the shell vial cell" and the "frog skin cell" as devised and described by Jacobs,[2] were extensively used. The following table, a result of a typical experiment with such "cells," shows that HCN undoubtedly acts in a manner quite similar to the two other acids mentioned.

Frog Skin Cell.

Reagent.	1 per cent HCN	$\frac{N}{100}$ KCN + HCN	$\frac{N}{100}$ NaOH + HCN	$\frac{N}{100}$ KCN + HCL	$\frac{N}{100}$ NaOH + HCL	Tap water.
pH of reagent.	5.5	7.2	7.2	7.2	7.2	7.2
Penetration (intracellular acidity).	++	++	++	+	–	–
Presence of intracellular CN.	++	++	++	+	–	–

This experiment, in which the reagents except HCN were all brought to the same pH value, as indicated in the table, seems to show rather clearly that HCN penetrates the cell and then ionizes, producing an intracellular acidity. That the membranes are not killed by these reagents is easily demonstrated by changing the reagents and substituting mineral acids and other reagents which are known not to penetrate living membranes in the above manner. No changes in intracellular acidity are noted in such check experiments.

The following table, giving the results of a typical experiment with the "frog skin cell" shows that an intracellular acidity can be produced by HCN in an alkaline medium.

Reagent.	HCN	CO_2 saturated H_2O	$\frac{N}{100}$ NaOH + HCN	$\frac{N}{100}$ KCN + HCN	$\frac{N}{1,000}$ KCN	Tap water.
pH of reagent.	6.4	3.8	7.6	7.5	9.5	7.3
Penetration (intracellular acidity).	++	++	++	+	Slight.	–

CO_2 in the concentration used penetrated in about the same relative time as the HCN. No exact quantitative comparisons in the relative rates of penetration of the two reagents have as yet been made. The extreme rapidity of penetration of HCN (in this experiment less than 1 to 2 minutes) must, however, be an important factor in explaining the rapid toxicity of the reagent.

Experiments carried out with bullfrog tadpoles, using the same solutions as in the "artificial cell" experiments, gave the same relative results. The animals were always killed first in those solutions in which the HCN penetrated the "frog skin cells" most quickly.

The results of these experiments show that in many respects HCN acts like CO_2 and H_2S. The experiments on protozoa seem to show quite definitely that HCN, like CO_2 and H_2S, acts from within the cell outward, while the "artificial cell" experiments tend to confirm the view that HCN, as a molecule and not in the ionized state, penetrates the cell. HCN penetrates the cell as a molecule and dissociates within the cell giving rise to H and CN ions. The acid effect within the cell is, of course, due to the H ions.

The importance of controlling the hydrogen ion concentration in experiments with solutions of KCN is thus apparent. This point has been recently indicated in a practical way by Edwards[5] who has shown that unneutralized solutions of KCN produce protoplasmic changes in ameba similar to those caused by solutions of KOH, while neutralized solutions of KCN produce changes of an entirely different nature.

SUMMARY AND CONCLUSIONS.

1. The physiological actions of HCN and its salts appear to be due (*a*) to the ease with which HCN molecules penetrate living cells and then ionizing, exert their influence by means of H ions and CN ions; (*b*) to the weakness of HCN as an acid, which permits at neutrality or at even slight alkalinity the presence of a considerable amount of free HCN molecules in the presence of their salts; (*c*) to specific effects occasioned by its chemical activity.

2. The order of resistance of various protozoans to HCN resembles that of the same protozoa to CO_2 and to H_2S, but is the reverse of their resistance to mineral acids.

3. HCN in acid, neutral, or slightly alkaline media produces intracellular acidity because of the rapid penetration of HCN molecules into the cell.

4. HCN acts specifically on certain species of protozoa, the order of resistance of *Paramecia* being *Paramecium caudatum* > *Paramecium multimicronucleatum* > *Paramecium aurelia* > *Paramecium bursaria*.

I am greatly indebted to the Fumigators Chemical Company, Inc., New York, who have placed at my disposal a generous supply of liquid hydrocyanic acid.

[5] Edwards, J. G., *J. Exp. Zool.*, 1923–24, xxxviii, 1.

THE COMBINATION OF SALTS AND PROTEINS. I.

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In most of the work on the properties of protein solutions it has been assumed that only the hydrogen and hydroxyl ions react with proteins to form complex ions and that salts are chemically inert. Loeb's experiments[1] on the effect of salts on the Donnan equilibrium show that in dilute protein and salt solutions any reaction between the salt ions and the protein is negligible compared with the reaction between protein and hydrogen and hydroxyl ions. There are indications, however, that other ions may combine with the protein since it has been found by Mellanby, Hardy, Pauli, Robertson, and others[2] that proteins affect the conductivity of salt solutions, and hence that there is presumably a compound formed between the salt and the protein. The marked effect of salts on the precipitation and solubility of proteins (Michaelis) also indicates that some reaction occurs with salts as well as with acids and bases. Kunitz[3] noted slight discrepancies in concentrated salt solutions. If this is actually the case, it would have a marked influence on the distribution of the ions of the salt when added to a system containing diffusible and non-diffusible ions separated by a membrane. It has been shown by Gibbs, and independently and in detail by Donnan, that at equilibrium the product of the activity of any pair of oppositely charged diffusible ions on one side of the membrane must be equal to the prod-

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

[2] For the literature on this subject see Robertson, T. B., The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918.

It has been found by Hastings in Van Slyke's laboratory that Ca protein salts are only partly dissociated, personal communication from Dr. Hastings.

[3] Kunitz, M., *J. Gen. Physiol.*, 1923–24, vi, 547.

uct of the activity of the same pair on the opposite side of the membrane. Expressed as an equation, this may be written

$$\frac{\left(A_o^{-n}\right)^{\frac{1}{n}}}{\left(A_i^{-n}\right)^{\frac{1}{n}}} = \frac{\left(B_i^{+m}\right)^{\frac{1}{m}}}{\left(B_o^{+m}\right)^{\frac{1}{m}}} = \ldots$$

in which A_i is the activity of an n-valent negative ion inside the membrane, A_o the activity of the same ion outside the membrane, and B is any positive ion. In dilute solution, the concentrations may be used instead of activities. It was shown by Procter and Wilson[4] that this equation held for the distribution of ions inside and outside of gelatin blocks in equilibrium with a solution of acid. Loeb[1] found that the hydrogen electrode potential between the inside and the outside solution was equal to the calomel electrode potential under the same conditions, and that therefore the ratio of the activities of the hydrogen ion must be equal to the reciprocal of the chlorine ion ratio, as is demanded by the theory. If the ions of a salt do not combine with the protein, the ratio of the total concentration of any cation as determined by analysis should be approximately the same as the ratio of the hydrogen ion activities corrected, of course, for the valence effect; whereas the ratio of the anion concentrations should be equal to the reciprocal of the hydrogen ion ratio. Loeb has shown that this is true for the chloride ion. For other ions, however, this relation has not been investigated in detail. In order to test this point a number of experiments were performed in which various salts were added to suspensions of gelatin particles and the suspension allowed to come to equilibrium at 0°. The solutions were then analyzed and the concentration ratios compared with the hydrogen ion activity ratios as determined with the hydrogen electrode. The results of some of these experiments are shown in Table I. It is evident that although, on the acid side at least, the concentration of the negative ions is approximately that expected, the ratios of the positive ion concentrations are very far from correct and may even be in the wrong direction. This effect is more marked with Ca and Zn than with K. If the concentrations are corrected to activities by means of Lewis

[4] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

TABLE I.

Distribution Experiment.

Comparison of Total Concentration Ratios with H^+ Activity Ratios.

Experiment.	Salt.	Concentration.	pH of gelatin.	H ratio: $\frac{\text{Filtrate}}{\text{Gelatin}}$.	Ion.	Ion ratio: $\frac{\text{Filtrate}}{\text{Gelatin}}$. Observed.	Ion ratio: $\frac{\text{Filtrate}}{\text{Gelatin}}$. Calculated from H ratio.
		M					
3/7	$CaBr_2$	0.005	2.83		Ca	1.84	8.4
	HBr	0.02		2.9	Br	0.446	0.344
3/14	KBr	0.004	3.75	3.63	K	3.55	3.63
	HBr	0.01			Br	0.26	0.275
	KBr	0.01	3.79	2.63	K	1.90	2.63
	HBr	0.01			Br	0.352	0.38
	KBr	0.02	3.81	1.66	K	1.34	1.66
	HBr	0.01			Br	0.475	0.60
3/19	$Ca(OH)_2$	0.00057	4.91		Ca	0.55	<1
	$CaBr_2$	0.004			Br	0.68	>1
	$Ca(OH)_2$	0.0027	5.84		Ca	0.32	<1
	$CaBr_2$	0.004			Br	0.91	>1
	$Ca(OH)_2$	0.0057	9.48		Ca	0.21	
	$CaBr_2$	0.004			Br	1.09	
	$CaBr_2$	0.004	4.79	0.76	Ca	0.75	0.58
	HBr	0			Br	0.59	1.32
	$CaBr_2$	0.004	4.31	1.90	Ca	2.57	3.6
	HBr	0.005			Br	0.41	0.52
	$CaBr_2$	0.004	2.42	2.3	Ca	2.35	5.3
	HBr	0.03			Br	0.465	0.43
	$CaBr_2$	0.004	1.95	1.6	Ca	2.15	2.56
	HBr	0.04			Br	0.50	0.62
	$CaBr_2$	0.004	1.70	1.4	Ca	1.93	1.96
	HBr	0.05			Br	0.56	0.71
3/27	$MgSO_4$	0.01	4.07	1.50	SO_4	0.51	0.45
	HBr	0.006			Br	0.69	0.66
					Mg	1.15	2.25

and Randall's ionic strength data,[5] the positive ion ratios become a little better but the negative ion ratios are worse. Evidently, either the Donnan equation does not hold under these conditions or a very appreciable amount of ions is combined with the protein. It will be noted that at pH 2.0 or lower, the calcium ratios become approximately correct. If the calcium combined with the same group as does the hydrogen, this would be the expected result since an excess of hydrogen ions would displace the calcium from its combination with the protein.

If these discrepancies are due to the combination of one of the ions of the salt with the protein then if the relative activities of the ions are determined by means of E.M.F. measurements, the correct values for the ratios should be obtained. Owing to the difficulty of obtaining satisfactory electrodes with alkaline earth or alkali metal electrodes, this experiment was performed with $ZnCl_2$.

Experimental.—40 gm. of dry powdered isoelectric gelatin were suspended in 500 cc. of solution containing the noted amounts of HCl and $ZnCl_2$, and stirred at 0° for 2 hours. Control experiments showed that this was sufficient time for equilibrium. The solution was then filtered and the filtrate analyzed for Zn and Cl. The volume of solution was noted and the concentrations of Zn and Cl in the gelatin were obtained by difference. The swollen gelatin was melted and the Zn, chloride, and hydrogen ion electrode potentials determined in the filtrate and melted gelatin at 25°, using a saturated KCl calomel electrode as a reference with a saturated KCl bridge and the usual potentiometric method. The Zn electrodes were prepared by amalgamating zinc rods. They gave satisfactory and reproducible results in aqueous solutions provided the solution was more acid than about pH 3.0. In the presence of gelatin the readings were constant without the addition of acid, but in acid solution the readings were low and increased slowly with time. This accounts for the discrepancy in the Zn potential in the acid solution. The chloride electrodes were prepared as described by MacInnes and Parker.[6] The electrode in the gelatin solution was negative to the electrode in the filtrate, in each case.

The activity of the ions in the solution, assuming no combination with the gelatin, was obtained from the total concentration by means of the table of Lewis and Randall,[5] connecting the ionic strength with

[5] Lewis, G. N., and Randall, M., Thermodynamics, New York and London, 1923, 382.

[6] MacInnes, D. A., and Parker, K., *J. Am. Chem. Soc.*, 1915, xxxvii, 1445.

the activity coefficient of the individual ions. It was assumed that the gelatin had no effect on the ionic strength but that the complex gelatin-hydrogen ion affected it in the same way as uncombined hydrogen ion. That is, the total HCl concentration was used to calculate the ionic strength and not simply the Cl concentration. It might be expected *a priori* that the protein would greatly affect the ionic strength of the solution, especially if, as seems probable, it is a polyvalent ion. It has been found, however, that the data are much more consistent if the assumptions mentioned above were used, *i.e.* that the pure protein has no effect and that the added HCl or other electrolyte affects the ionic strength in the same way as it would in the absence of gelatin. This amounts to assuming that the complex protein-hydrogen ion is monovalent.

The results of the experiment are given in Table II. The various electrode potentials agree within 1 mv., except in the most acid solution. In this case, as was stated above, the Zn electrode potential in the presence of gelatin is not constant. The ratios of the total concentrations of chloride ion are also quite close to the ion activity ratios, but the ratios of the total hydrogen ion and the total Zn ion concentrations are much too small; *i.e.*, the activities of the zinc and hydrogen ions in the gelatin are much smaller than would be expected from their total concentrations. The experiment shows directly, therefore, that the gelatin decreases the activity of the hydrogen and Zn ions but has little effect on that of the chloride ion. In the strongly acid solution there is no effect on the zinc ion when the correction for the ionic strength of the solution is made. These two facts render it very unlikely that the effect of the gelatin is upon the activity coefficient of the ions, since it would be expected that this would be apparent in all cases, but show that it is rather upon the concentration of the ions. That is, the hydrogen ion and the zinc ion combine with the gelatin to form complex ions which do not influence the electrodes. This has been well established in the case of hydrogen ions, and the present experiment shows that zinc behaves in an analogous way but to a smaller extent. The dissociation of the complex gelatin-Zn ion is evidently much greater than that of the gelatin-hydrogen ion.

The agreement between the various electrode potentials shows that the system obeys the Donnan equilibrium quite closely, and that the discrepancies obtained when total concentrations are compared are due to the formation of compounds between the gelatin and the ions.

This furnishes a method for the determination of the formation of such complexes without the use of E.M.F. measurements. The combination of any ion with a protein can be easily determined even though

TABLE II.

Comparison of Activity Ratios from Electrode Potential Measurements with Total Concentration Ratios. 40 Gm. of Gelatin. Total Volume 1,000 Cc. Total $ZnCl_2$ Concentration 0.01 M.

Experiment		1	2	3
Total HCl concentration		0.0105	0.0200	0.04
pH of gelatin		4.05	3.51	2.18
H electrode E.M.F., *mv*		15.5	20.0	14.5
Ag-AgCl electrode E.M.F., *mv*		15.2	19.0	15.0
Zn electrode E.M.F., *mv*		14.8	18.0	10.0
Ratio: $\frac{\text{Activity H in filtrate}}{\text{Activity H in gelatin}}$		1.83	2.19	1.76
Ratio: $\frac{\text{Total conc. H in filtrate (as HCl)}}{\text{Total conc. H in gelatin (as HCl)}}$		0.017	0.018	0.166
Ratio: $\frac{\text{Activity Cl in filtrate}}{\text{Activity Cl in gelatin}}$	calc. from H ratios	0.546	0.457	0.568
	" " observed E.M.F.	0.552	0.48	0.555
	" " analysis	0.508	0.42	0.48
Ratio: $\frac{\text{Total conc. Cl in filtrate}}{\text{Total conc. Cl in gelatin}}$		0.488	0.41	0.46
Ratio: $\frac{\text{Activity Zn in filtrate}}{\text{Activity Zn in gelatin}}$	calc. from H ratios	3.35	4.8	3.1
	" " observed E.M.F.	3.18	4.08	2.2
	" " analysis	2.11	3.23	3.0
Ratio: $\frac{\text{Total Zn conc. in filtrate}}{\text{Total Zn conc. in gelatin}}$		1.85	2.68	2.44

it is not possible to have an electrode for that ion. It is only necessary to determine the distribution in a system such as the above and also the activity ratio for some ion, such as hydrogen or chloride, which can be conveniently measured by E.M.F. measurements. Since the activity ratios of the other ions must be related to that of the chloride or hydrogen as expressed by Donnan's equation, the activity of the ion in the protein solution can be calculated from that of the outside solution. Lewis and Randall's tables of activity coefficients at

various ionic strengths furnish the data for determining this when the total concentration and ionic strength are known. The activity of the ion in the protein solution divided by its activity coefficient for a solution of the proper ionic strength gives the total concentration of the ion,[7] and this value subtracted from the total concentration as determined by analysis gives the amount combined with the protein.

The results of several experiments carried out in this way with $ZnCl_2$ are shown in Table III. They show that in 15 per cent gelatin at pH 4.7, over 50 per cent of 0.01 M Zn is combined with the gelatin and about 10 per cent of the chloride. It will be noted that in the first experiment the amount of Zn actually removed by the gelatin from the solution is just equivalent (1/2) to the chloride removed, since the outside solution must remain electrically neutral. If the particles were filtered, dried, and analyzed, it would be found, therefore, that they contained equivalent amounts of Zn and Cl and hence it might be concluded that the protein combined with both ions of the salt. This is, however, incorrect. Practically none of the chloride is combined and the remainder is simply held by electrostatic attraction. This illustrates the practical impossibility of obtaining reliable results as to compound formation by the analysis of protein precipitates. As the solution is made progressively more acid, less and less Zn is combined until at pH 2.0 there is practically none combined. The amount of chloride combined on the other hand increases about in proportion to the concentration, so that the per cent combined remains constant. The result bears out the idea, expressed before, that the Zn combines with the same group as does the hydrogen.

[7] This calculation necessarily involves a relation between the electrode potential of an ion and its concentration in the presence of other electrolytes. This is at present a very uncertain step, but it is possible to make the calculation by means of Lewis' ionic strength data. This method has been used throughout the present paper, although it is realized that such calculations are difficult for simple physical interpretation. The physical significance of the value obtained by dividing the activity of the Zn ion by its activity coefficient (as given by Lewis for a solution of that particular ionic strength) may be best expressed as the concentration of $ZnCl_2$ which would give the observed electrode potential in a solution of the same ionic strength. Since the amount of zinc found by analysis is in excess of this it seems logical to suppose that the excess of zinc is combined with the protein.

Determination of the Combination of Gelatin with Zn Chloride by Direct E.M.F. *Measurements.*

In order to confirm the results obtained by this method a series of experiments was made to determine the effect of gelatin on the potential of a zinc or chloride electrode in zinc chloride solution.[8] The

TABLE III.

Calculation of Zn and Cl Combined with Gelatin from Cl Activity Ratios. Total $ZnCl_2$ Concentration 0.01 M.

Experiment	1	2	3	4
pH of gelatin	4.7	4.05	3.51	2.18
Gm. of dry weight of gelatin per 100 cc. of swollen gelatin	19	14	11	9
Total concentration Zn in filtrate, M	0.0082	0.0115	0.013	0.014
" " Cl " " M	0.0178	0.0236	0.027	0.040
" " H " " M		0.0006	0.001	0.012
μ (ionic strength) of filtrate	0.025	0.035	0.039	0.053
γ Zn in filtrate	0.50	0.48	0.47	0.43
α Zn^{++} in filtrate	0.0042	0.0055	0.0060	0.0060
Ratio: $\frac{\alpha \text{ Cl in filtrate}}{\alpha \text{ Cl in gelatin}}$ (from Cl P.D.)	0.813	0.552	0.48	0.555
α Zn in gelatin (calculated)	0.0028	0.0018	0.0014	0.0018
Total H^+ in gelatin		0.036	0.055	0.075
" Zn " " observed	0.0126	0.0062	0.0048	0.0056
" Cl " " "	0.0253	0.0483	0.065	0.086
μ in gelatin	0.038	0.054	0.070	0.091
γ Zn in gelatin	0.47	0.42	0.39	0.35
C_{Zn} " " (calculated)	0.0059	0.0043	0.0036	0.0052
$C_{Zn\text{-gelatin}}$	0.0067	0.0019	0.0012	0.0004
Per cent Zn as Zn-gelatin	54	31	26	7
γ Cl in filtrate	0.88	0.86	0.86	0.84
α Cl " "	0.0156	0.020	0.023	0.0333
α Cl " gelatin (calculated)	0.0192	0.037	0.0466	0.060
γ Cl " "	0.86	0.83	0.82	0.80
C_{Cl^-} " "	0.0224	0.044	0.057	0.075
$C_{\text{gelatin-Cl}}$	0.003	0.0013	0.008	0.011
Per cent Cl as gelatin-Cl	11	9	12	12.5

zinc and Ag-AgCl electrodes were first standardized against a series of concentrations of zinc chloride made up in 0.001 M HCl, and the

[8] Measurements of Zn potentials in $ZnSO_4$-gelatin solutions have been made by Mutscheller (Mutscheller, A., *J. Am. Chem. Soc.*, 1920, xlii, 2142) who found that some Zn^+ but more SO_4^- was combined with the gelatin.

standard electrode potentials were calculated by using Lewis and Randall's figures for the ionic strength μ, and the activity coefficient γ. The values for the E.M.F. of the cell

Zn | M $ZnCl_2$ | saturated KCl | HgCl | Hg

and for the cell

Ag | AgCl | M $ZnCl_2$ | saturated KCl | HgCl | Hg

are shown in Table IV. All measurements were made at 25°. The experiment shows that the values obtained for the standard molar Zn

TABLE IV.

Zn-ZnCl₂ E.M.F. Measurements.

Concentration $ZnCl_2$.	Zn μ	Zn γ	$\gamma m = \alpha Zn$	pZn	$E°$ observed.	$E° = E$ obs. $-$ (0.0295 pZn)
M						
0.05	0.15	(0.32)	0.0162	1.79	1.0548	(1.002)
0.02	0.06	0.41	0.0082	2.086	1.063	1.0025
0.01	0.03	0.50	0.0050	2.301	1.070	1.002
0.005	0.015	0.57	0.00285	2.545	1.077	1.002
0.002	0.006	0.64	0.00128	2.893	1.087	1.0016
						Average......1.0020
						Sat. KCl—H_2.0.243
						Zn, Zn^{++}; $E°$=0.7590

Ag-AgCl-ZnCl₂ E.M.F. Measurements.

Concentration Cl.	Cl μ	Cl γ	$\gamma m = \alpha Cl$	pCl	E observed.	$E° = E$ obs. + (0.059 pCl)
M						
0.041	0.060	0.83	0.0341	1.467	−0.070	0.0165
0.021	0.030	0.87	0.0183	1.737	−0.0850	0.0170
0.011	0.0155	0.90	0.0099	2.004	−0.1002	0.0176
0.005	0.0065	0.94	0.0047	2.328	−0.1190	0.017
						Average.......0.017
						Sat. KCl − H_2.0.243
						Ag, AgCl, Cl^-; $E°$ = −0.226

or Ag electrode against the saturated KCl calomel electrode are constant and independent of the salt concentration. The saturated calomel electrode used gave a calculated E.M.F. of $E = -0.243$ against

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$$Zn \mid M\ ZnCl_2 \mid \text{saturated } KCl \mid HgCl \mid Hg$$

and for the cell

$$Ag \mid AgCl \mid M\ ZnCl_2 \mid \text{saturated } KCl \mid HgCl \mid Hg$$

are shown in Table IV. All measurements were made at 25°. The experiment shows that the values obtained for the standard molar Zn

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0.005	0.015	0.57	0.00285	2.545	1.077	1.002
0.002	0.006	0.64	0.00128	2.893	1.087	1.0016
						Average......1.0020
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						$Zn, Zn^{++}; E°$=0.7590

Ag-$AgCl$-$ZnCl_2$ E.M.F. Measurements.

Concentration Cl.	Cl μ	Cl γ	$\gamma m = \alpha Cl$	pCl	E observed.	$E° = E$ obs. + (0.059 pCl)
M						
0.041	0.060	0.83	0.0341	1.467	−0.070	0.0165
0.021	0.030	0.87	0.0183	1.737	−0.0850	0.0170
0.011	0.0155	0.90	0.0099	2.004	−0.1002	0.0176
0.005	0.0065	0.94	0.0047	2.328	−0.1190	0.017
						Average.......0.017
						Sat. KCl − H_2.0.243
						$Ag, AgCl, Cl^-$; $E°$ = −0.226

or Ag electrode against the saturated KCl calomel electrode are constant and independent of the salt concentration. The saturated calomel electrode used gave a calculated E.M.F. of E = −0.243 against

the normal hydrogen electrode, so that the value obtained for the standard Zn, Zn^{++} is 0.759 volt, and for the standard Ag, AgCl, Cl^- is − 0.226, which are within 1 mv. of the values given by Lewis and Randall.[9] This shows that the electrode measurements are reliable, and also that the data for the activity coefficients given by Lewis and Randall apply to $ZnCl_2$ solutions with considerable accuracy.

The experiments were then repeated with the addition of increasing concentrations of isoelectric gelatin. The results are given in Table V. The amount of Zn combined increases steadily and in the presence of 15 per cent gelatin amounts to 64 per cent. This agrees quite well

TABLE V.

Effect of Increasing Concentration of Gelatin on Zn and Chloride Activity of 0.01 M $ZnCl_2$.

Concentration of gelatin.	Zinc (γ = 0.50).					Chloride (γ = 0.87).				
	E.M.F. Zo	αZn	C_{Zn}	C_{Zn} combined with gelatin.	Per cent Zn combined.	E.M.F.	αCl	C_{Cl}	C_{Cl} combined.	Per cent Cl combined.
per cent								M		
0	1.070	0.0050	0.010	—	—	0.084	0.0183	0.021	—	—
1	1.072	0.00426	0.0085	0.0015	15	0.084	0.0183	0.021	—	—
2	1.073	0.00398	0.0078	0.0022	22	0.082	0.0183	0.021	—	—
5	1.076	0.00311	0.0062	0.0038	38	0.084	0.0183	0.021	—	—
10	1.081	0.00211	0.0042	0.0058	58	0.0852	0.0180	0.0201	0.0003	1
15	1.083	0.0018	0.0036	0.0064	64	0.0860	0.0172	0.0198	0.0012	6

with the results obtained from the distribution experiment (Table III). The amount of chloride combined is below the accuracy of the experiment until high concentrations of gelatin are reached, and the amounts to about 6 per cent.

The experiment was repeated using 10 per cent gelatin and differe concentrations of $ZnCl_2$. The results are shown in Table VI. would be expected and as is the case with acids, the amount combi increases with increasing Zn concentration but the per cent combi decreases.

The preceding experiments show that $ZnCl_2$ combines with gel in the same way as hydrogen chloride, but to a less extent. T

[9] Lewis and Randall,[5] pp. 407, 420.

seems no reason to suppose that this is other than ordinary complex ion formation; but, as in the case of hydrogen, experimental proof of the stoichiometric nature of the reaction is difficult to obtain. It can be shown, however, that the reaction is rapidly and completely reversible, which is the result expected if the compound is an ordinary chemical one and is the opposite of the result obtained in cases such as that of charcoal where there is surface adsorption. Table VII

TABLE VI.

Combination of Zn with Gelatin from Measurements with Zn Electrodes. 10 Per Cent Gelatin.

Concentration $ZnCl_2$.	E.M.F.	a_{Zn}	C_{Zn}	C_{Zn} combined with gelatin.	Per cent Zn combined.
M					
0.05	1.0595	0.011	0.0345	0.0155	31
0.02	1.0718	0.0043	0.0105	0.010	50
0.01	1.0812	0.0021	0.0042	0.0058	58
0.005	1.097	0.00095	0.00167	0.00383	76

TABLE VII.

Reversibility of Zn-Gelatin Combination.

	A	B
Method of preparing solution....................	50 cc. $\frac{M}{50}$ $ZnCl_2$ + 50 cc. 10 per cent isoelectric gelatin, 1 hr., 25°.	1 cc. $\frac{M}{1}$ $ZnCl_2$ + 25 cc. 20 per cent gelatin, 1 hr., 25° and then diluted to 100 cc.
E.M.F., Zn electrode........	1.0760	1.0760
pH.....................	4.70	4.70

shows that the activity of Zn^{++} and of H^+ are the same in the presence of the same concentration of gelatin whether the Zn and gelatin were mixed in concentrated solution and then diluted or whether they were diluted first.

Effect of the Combination with the Salt on the Properties of the Protein Solution.

If the gelatin combines more with one ion of the salt than with the other, as is indicated by the preceding experiments, and if the resulting compound is itself an ion, then the addition of $ZnCl_2$ to isoelectric

gelatin must give rise to ionized gelatin and hence to a Donnan equilibrium just as does the addition of acid, except to a much smaller extent. The osmotic pressure, swelling, and viscosity of gelatin should all be affected. That this is actually the case may be seen from Tables VIII, IX, and X. The effect is small, and in dilute gelatin solution

TABLE VIII.

Effect of $ZnCl_2$ on Viscosity of Gelatin Solutions.
5 Per Cent Isoelectric Gelatin.

Conc. $ZnCl_2$, M.	0.5	0.10	0.01	0
pH	3.95	4.33	4.55	4.8
Relative viscosity compared to corresponding aqueous solution of $ZnCl_2$ after 24 hrs. 37°	6.31	7.86	7.84	7.15

TABLE IX.

Effect of $ZnCl_2$ on Swelling of Particles of Isoelectric Gelatin.
8 Gm. of Dry Isoelectric Gelatin Added to 100 Cc. of $ZnCl_2$ and Kept at 2°C. for 18 Hours. Total Volume 106 Cc.

Conc. $ZnCl_2$, M.	0.5	0.10	0.01	0
Volume of filtrate (average of 3 experiments)	49	53	55	59
" " gelatin	57	53	51	47

TABLE X.

Effect of $ZnCl_2$ on Osmotic Pressure of Gelatin. 25 Cc. of 10 Per Cent Isoelectric Gelatin in Collodion Sac Suspended in 60 Cc. of Various Concentrations of $ZnCl_2$ pH 4.7 at 37°. Readings after 24 Hours.

Conc. $ZnCl_2$, M.	0	0.02	0.20	0.50
Osmotic pressure in mm. Hg (average of 6 experiments)	37.7±1.0	56±1.8	53.0±0.8	48.2±0.6
Chloride ratio (from E.M.F.): $\frac{\alpha \text{ Cl inside}}{\alpha \text{ Cl outside}}$		1.20		
αZn^{++} ratio, calculated from Cl ratio		0.70		
αZn^{++} " " " total Zn concentration		1.20		

would be negligible compared to the effect of acid. The fact that the Zn is more concentrated inside the membrane than outside shows, however, that the result is not due to the H^+, since if the Zn were merely inert the concentration of Zn outside would necessarily be greater than inside in order to agree with the H^+ and Cl^- ratios.

The osmotic pressure experiment is the most direct, since both the swelling and viscosity are influenced by other factors than the ion concentration. The swelling depends also on the "cohesion" of the gelatin particles; if the force which tends to hold the particles to the original size should decrease this swelling will evidently increase although the osmotic pressure may be no greater. This presumably accounts for the fact that the swelling maximum does not agree with the maximum of osmotic pressure.

The viscosity is a function of time and the selection of the proper value is difficult. The figures given were obtained after 24 hours at 34° and were constant for about 10 hours. Before this time, the control and most concentrated solutions decreased in viscosity while the intermediate concentrations increased.

It will be noted in Table VIII that the pH of the solutions becomes lower as Zn is added, although both solutions were originally pH 4.7. Diluting either the gelatin or $ZnCl_2$ with water does not cause a change in pH. The effect is therefore due to a reaction between the $ZnCl_2$ and gelatin. If, as was suggested, the Zn replaces the H from combination this increase in acidity is due to the liberated H^+ just as adding acid was found to increase the Zn^{++} concentration.

SUMMARY.

1. It has been found that the ratios of the total concentrations of Ca, Mg, K, Zn, inside and outside of gelatin particles do not agree with the ratios calculated according to Donnan's theory from the hydrogen ion activity ratios.

2. E.M.F. measurements of Zn and Cl electrode potentials in such a system show, however, that the ion activity ratios are correct, so that the discrepancy must be due to a decrease in the ion concentration by the formation of complex ions with the protein.

3. This has been confirmed in the case of Zn by Zn potential measurements in $ZnCl_2$ solutions containing gelatin. It has been found that in 10 per cent gelatin containing 0.01 M $ZnCl_2$ about 60 per cent of the Zn^{++} is combined with the gelatin.

4. If the activity ratios are correctly expressed by Donnan's equation, then the amount of any ion combined with a protein can be determined without E.M.F. measurements by determining its distribution

in a proper system. If the activity ratio of the hydrogen ion and the activity of the other ion in the aqueous solution are known, then the activity and hence the concentration of the ion in the protein solution can be calculated. The difference between this and the total molar concentration of the ion in the protein represents the amount combined with the protein.

5. It has been shown that in the case of Zn the values obtained in this way agree quite closely with those determined by direct E.M.F. measurements.

6. The combination with Zn is rapidly and completely reversible and hence is probably not a surface effect.

7. Since the protein combines more with Zn than with Cl, the addition of $ZnCl_2$ to isoelectric gelatin should give rise to an unequal ion distribution and hence to an increase in swelling, osmotic pressure, and viscosity. This has been found to be the case.

OBSERVATIONS ON THE INJURIOUS EFFECTS OF HIGH TEMPERATURES ON FROG SKIN.

By JOHN W. KLOPP.

(*From the Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, and the Marine Biological Laboratory, Woods Hole.*)

(Accepted for publication, June 22, 1924.)

It has been shown by Osterhout (1) that the electrical conductance of living tissues may be used as a general criterion of their vitality, and may in particular be employed in quantitative studies of injuries inflicted upon them. It has been found by this investigator that such injuries may give rise to one of three results, namely (*a*) progressive change leading to death, (*b*) partial recovery, or (*c*) complete recovery. These studies of Osterhout suggest the possibility of using in a similar way some other electrical property of tissues in attempting to define such indefinite terms as vitality, injury, and death. Thus, for example, it has been known since the early work of Du Bois-Reymond (2) that there is a difference of electrical potential between the inner and outer surfaces of the skin of the frog; and this potential difference has been the subject of much investigation since that time.[1] The question naturally arises how far the results of Osterhout might be paralleled by those obtained by studies on it; and the observations here reported were made with the object of determining this point.

The method employed was as follows: The skin, having been removed from various parts of the pithed animal with a minimum of trauma, was cut into squares of sufficient size to fit without undue stretching over the flanged end of a short glass cylinder of about the caliber of an ordinary test-tube, and was held in place there by a rubber band. The tube was then filled with Ringer's solution, and its skin-covered end immersed in a dish of the same solution. Non-polarizable Zn-$ZnSO_4$ electrodes served as leads from each of these two solutions, and the

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[1] More extensive references to the literature on this and certain other points will be given when the complete data are published in a later paper.

difference of potential between them was measured by means of an accurate potentiometer. Precautions were taken to see that the two electrodes themselves were at the same potential at the beginning and during the course of the experiments.

Measurements made in the manner described have confirmed the observations of others that when Ringer's solution is used on both sides of the skin, the external surface is always electronegative to the internal, and none of the procedures used in these experiments sufficed to reverse this polarity. The skin of the large bullfrog (*Rana catesbiana*) obtained at Woods Hole showed considerably higher P.D. than those recorded by most previous observers who have usually obtained values from 30 to 90 mv. The present observations on freshly captured bullfrogs gave a range of from 55 to 141 mv., though values of the usual magnitude were obtained with ordinary laboratory frogs in Philadelphia.

That there is some correlation between the vitality of the skin and its observed P.D. is indicated in a general way by the fact that sickly frogs and those held captive a long time usually give much lower values than do healthy ones recently captured. Similarly, it is easy to show that various noxious agents which are generally admitted to damage the skin (*e.g.* drying, formalin, alcohol, strongly hypertonic salt solutions, etc.) cause a progressive drop in the P.D., leading, finally, to a zero potential. While it is thus possible to use for purposes of study a variety of injurious agents, high temperatures alone were employed in these experiments because of the ease with which they can be controlled and measured.

The nature of the effects produced by continuous exposures to high temperatures was first studied, and the results from one typical experiment are reproduced in Fig. 1. For purposes of more ready comparison the readings have been changed from millivolts to percentages of their value at the beginning of the experiment. This device was utilized because it usually is not possible to obtain, even from the same animal, a number of pieces of skin the P.D. of which are exactly the same. It will be noted that the control curve for room temperature follows a somewhat erratic course (see also Fig. 2). This is typical; and the most common result is a more or less regular fall in the P.D. toward zero. Usually, with the skin on the tubes in

Ringer's solution, this point has not been reached in 24 hours; and the skin may be kept for this length of time on the dead animal without entirely losing its P.D. if precautions are taken to keep it moist.

It will be observed that with the exception of the one representing the lowest temperature, where there was a short characteristic preliminary rise, the curves all show a progressive fall, rapid at first, but becoming slower and slower as the zero point is approached.

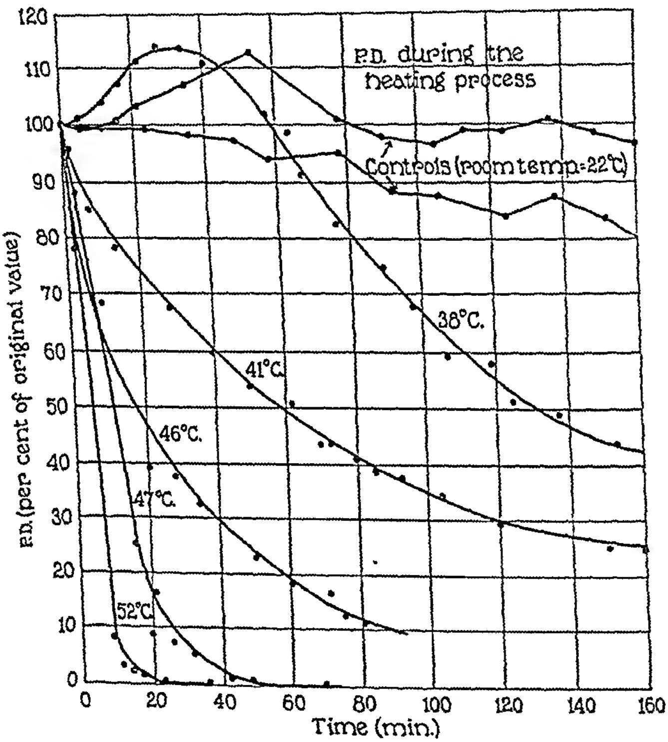

FIG. 1. Course of the P.D. of frog skin during continuous exposure to injurious temperatures. Each curve represents a single experiment.

In certain cases a good straight line relation may be obtained by plotting the logarithm of the P.D. (measured in per cent of its original observed value in millivolts) against time; but the author does not wish to draw the conclusion that a monomolecular reaction is necessarily involved, since many purely physical processes are known to follow the same law. Brooks (3) and others have warned against the mistaken resemblance of the time curves of a number of destruc-

tive biological processes to that for monomolecular reactions, pointing out that the apparent logarithmic straight lines are in reality S-shaped curves. Some of the logarithmic curves obtained from the present experiments show this latter relation. A number of attempts have been made to determine in an exact manner a temperature coefficient for the injurious effects of heat by comparing the times required for different temperatures to depress the P.D. to a given level (*e.g.* 50 per cent of the original); but these attempts were abandoned because of the great variability of the material. It was found, however, that such temperature coefficients as could be obtained were invariably much higher than those characteristic of ordinary chemical reactions, and were of the same general order of magnitude as those found, for example, by Chick and Martin (4) for the coagulation of proteins, and by various observers for the destruction of living cells (5).

Having determined the effects of continuous exposures to high temperatures, experiments were then performed to study the after-effects produced by short exposures of various lengths. The general procedure was to measure the P.D. of the skin as usual in Ringer's solution at room temperature for 30 to 40 minutes, to establish the normality of the specimen, subsequently immersing it in a hot Ringer bath at the desired temperature. As soon as this temporary exposure was completed the skin was replaced in the original Ringer's solution, and measured at room temperature from that time on. From these experiments a few typical ones are selected to illustrate the nature of the effects produced (Fig. 2). It will be seen that by varying the intensity of the injury it is possible to obtain all three types of response described by Osterhout, namely (*a*) progressive decline to death, (*b*) partial recovery, and (*c*) complete recovery. It is impossible in a figure of this size to continue the curves beyond a point representing 200 minutes from the beginning of the experiment, but the author has followed such experiments for many hours without obtaining further changes of significance. In addition to the method thus described another has been used in some experiments, namely varying the time of the exposure with the temperature constant, instead of using the temperature as the variable. The results, while somewhat less regular than those already described, nevertheless have been of the same nature.

The results obtained, therefore, parallel in a general way those of Osterhout. The P.D. of frog skin may to a certain extent be used as a criterion of injury, and from measurements of its magnitude at intervals curves of recovery and death may be constructed. It must be admitted, however, that for purposes of quantitative study involving the exact nature of the processes of death and recovery the method is

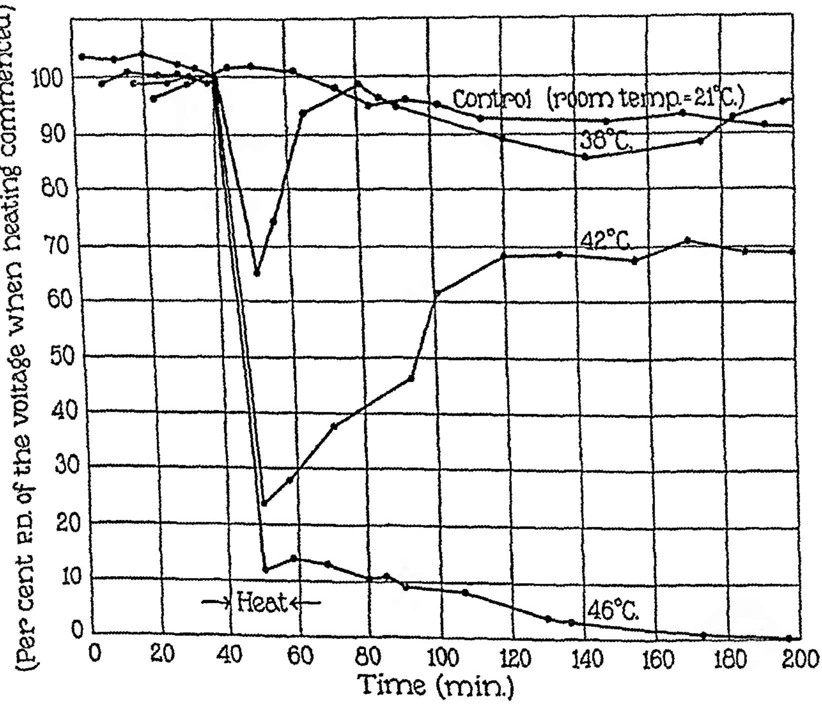

FIG. 2. Showing the after-effects of temporary exposure to injurious temperatures for 10 minutes. (Heat applied from the 40th to 50th minutes.) Each curve represents a single experiment.

inferior, at least in the hands of the author, to the electrical conductance method of Osterhout. By reason of its more limited applicability, and because of the greater complexity and variability of the material, the method herein described yields results which are not easily reproducible; though it may perhaps lay some claim to value as an independent and supplementary means of testing the conclusions of previous workers.

of the tablets, when this content is reasonably close.

2. The degree of salicylate or of acetophenetidin absorption is of a wide variation within the same individuals for the same product upon different days, and is probably governed by several physiologic factors as well as the composition and physical characteristics of the tablets.

3. It is possible to demonstrate salicylate in the plasma within one to two minutes after ingestion of tablets containing aspirin at a dose level of seven to ten grains.

4. *p*-Aminophenol is not present in the blood within one to two minutes, but does occur in about fifty per cent of the subjects within eight minutes.

5. The data observed are treated statistically to aid in evaluating the widely spread individual figures

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Analogs of Nucleotides III*

Syntheses in the Series of Adenosine Phosphonate Derivatives

By MANFRED E. WOLFF† and ALFRED BURGER

The synthesis of 9-[5′-deoxy-5′-(diethyl phosphonate-β-D-ribofuranosyl)]-6-amino-2-(methylthio)purine is described. Methyl 2,3-isopropylidene-5-deoxy-5-(diethyl phosphonate)-D-ribofuranoside was converted to the aldehydo triacetate in several steps. Treatment with 2-(methylthio)-4,6-diaminopyrimidine gave a Schiff base which was transformed to the N-ribofuranoside, coupled with 2,5-dichlorophenyldiazonium sulfate and reduced. Thioformylation and cyclization to the purine gave the final product.

THE SIGNIFICANCE of the C-O-P moiety in adenosine phosphate with respect to the various reactions in which this metabolite is involved should be capable of examination by biochemical evaluation of adenosinephosphonic acid and the esters and anhydrides derived from it. The C-P bond in such phosphonic acid analogs would not be subject to hydrolysis, and its degradation, if any, would occur at a rate of much smaller magnitude than is found in adenosine phosphate. The phosphonic acid analogs are, therefore, potential adenosine phosphate antagonists since they could block essential cellular receptor sites by virtue of their pronounced structural similarity to the natural phosphate factors.

The preparation of phosphonoglucopyranosyl derivatives of adenine as model compounds for syntheses involving phosphonoribofuranosyl groups has been described by us in an earlier study (1). This paper is a progress report on experiments designed to furnish ultimately adenosine-5′-phosphonic acid.

The synthetic route employed for the preparation of the glucopyranosyl derivatives involved the treatment of a 6-acylamido-9-(2′,3′,4′-tri-O-acetyl-6′-deoxy-6′-bromo-β-D-glucopyranosyl) purine with a trialkyl phosphite in a Michaelis-Arbuzov transformation. By analogy, the most obvious preparation of an adenosine-5′-phosphonic acid derivative would involve the similar utilization of a 5′-deoxy-5′-haloadenosine derivative. These halonucleosides do not exist as such, however, but in the form of cyclonucleoside quaternary salts (2). It was necessary, therefore, to use a synthetic scheme in which a suitably protected 5-ribosephosphonate fragment could be introduced into a second moiety which could then be converted into an adenine structure.

Among the methods which satisfy these requirements are (*a*) the glycosidation of an imid-

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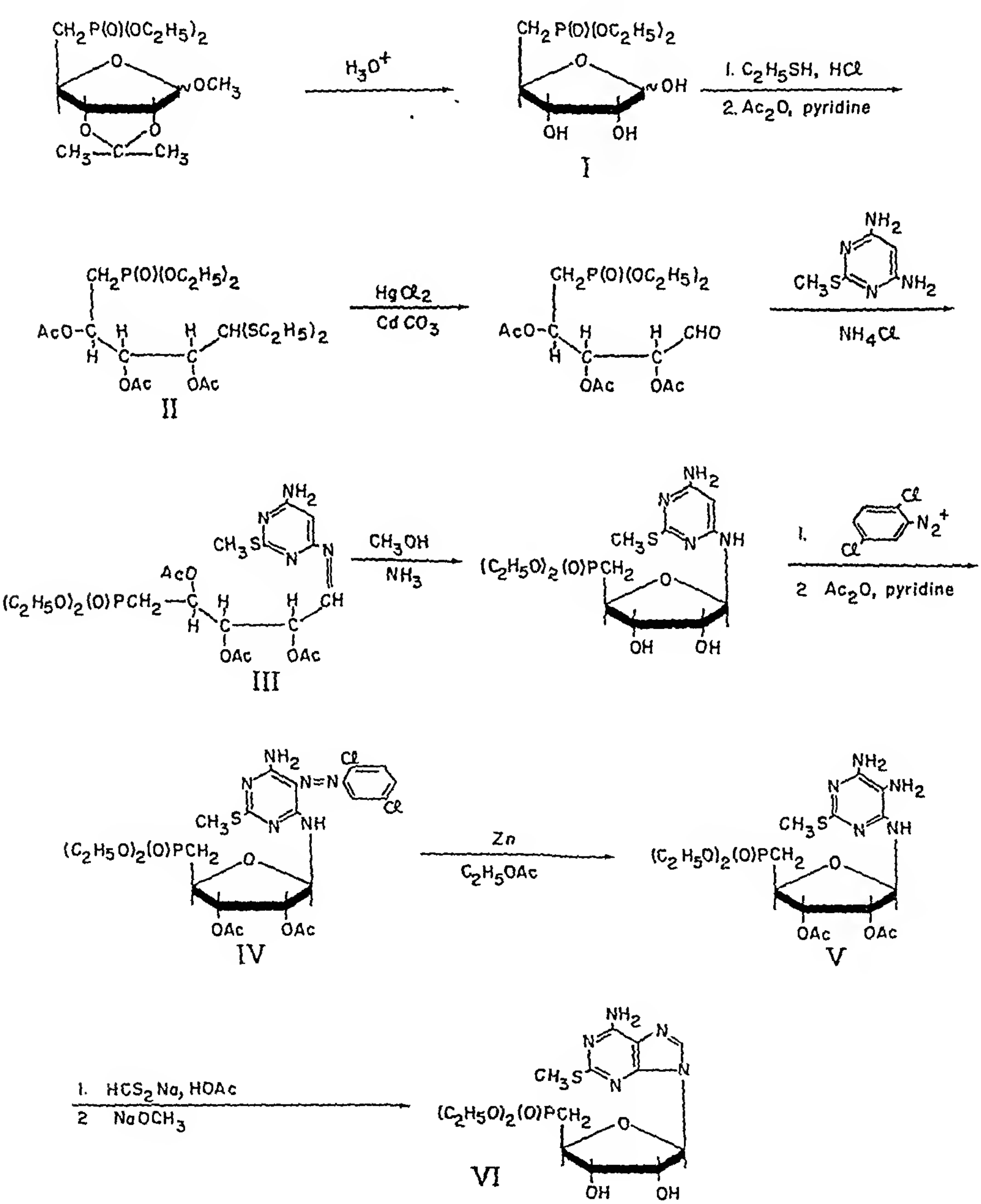
azole derivative followed by cyclization to a purine (3), or (b) the ribosidation of a pyrimidine derivative followed by cyclization to a nucleoside (4). The latter method was selected in the present synthesis.

D-Ribose was converted in four steps to methyl 2,3-isopropylidene-5-deoxy-5-(diethyl phosphonate)-D-ribofuranoside (1). The masking groups were removed by acid hydrolysis and the 5-deoxy-5-(diethyl phosphonate)-D-ribofuranose (I) was characterized as its osazone. Treatment with ethanethiol in hydrochloric acid medium and subsequent acetylation gave 2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose diethyl mercaptal (II) which was purified by molecular distillation or chromatography on alumina. Removal of the mercaptal groups with mercuric chloride regenerated the aldehyde function, and the product was condensed with 2-(methylthio)-4,6-diaminopyrimidine to give the corresponding Schiff base (III).

For the introduction of the 5-amino function into the pyrimidine, coupling with 2,5-dichlorophenyldiazonium sulfate according to Todd's



method (5) was chosen in order to avoid destruction of the ribosidic linkage. The Schiff base (III) was deacetylated with methanolic ammonia, coupled, and the resulting azo dye was purified by adsorption on chromatographic alumina and elution through conversion to the acetylated derivative (IV). Zinc dust reduction cleaved the azo group to produce 5,6-diamino-4-(2',3'-di-O-acetyl-5'-deoxy-5'-(diethyl phosphonate)-D-ribofuranosylamino) - 2 - (methylthio)pyrimidine (V). Although this compound was not obtained in a state of complete analytical purity, it was thioformylated with dithioformic acid, deacylated, and cyclized to furnish 9-[5'-deoxy-5'-(diethyl phosphonate)-β-D-ribofuranosyl]-6-amino-2-(methylthio)purine (VI) which was characterized as the chloroplatinate.

EXPERIMENTAL

5-Deoxy-5-(diethyl phosphonate)-D-ribose.—A stirred suspension of 33.2 Gm. (0.102 mole) of syrupy methyl 2,3-isopropylidene-5-deoxy-5-(diethyl phosphonate)-D-ribofuranoside (1) in 300 cc. of 0.4 *N* sulfuric acid was heated on the steam bath for five hours. The resulting orange-brown solution was filtered from a small amount of insoluble oil, neutralized to pH 7 with barium carbonate, filtered, and partially decolorized with Darco. The filtered solution was evaporated at 50° to give 24.8 Gm. (90%) of a thick dark syrup which did not crystallize.

A sample (500 mg.) was dissolved in 4 cc. of water, 1.2 Gm. of phenylhydrazine in 6 cc. of 50% acetic acid was added, and the solution was heated on the steam bath (6). The solution set to a solid mass after five minutes, 30% acetic acid was added to cover the crystals, and heating was continued for forty minutes. The canary-yellow osazone was isolated by filtration, washing with 30% acetic acid and water, and recrystallization from absolute alcohol, m. p. 194–196° (decompn.).

Anal.—Calcd. for $C_{21}H_{29}N_4O_5P$: C, 56.24; H, 6.52. Found: C, 55.87; H, 6.70.

2,3,4-Tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose diethyl mercaptal.—A stirred, ice-cold solution of 55.5 Gm. (0.205 mole) of 5-deoxy-5-(diethyl phosphonate)-D-ribose in 75 cc. of concentrated hydrochloric acid was treated with 100 Gm. of ethyl mercaptan in 4 portions. The mixture was then stirred at 0° for ten minutes, at 27° for fifteen minutes, and at 40° for ten minutes. It was poured carefully into 800 cc. of saturated sodium bicarbonate solution with stirring, and the pH adjusted to 7 with solid sodium bicarbonate. The filtered solution was extracted with 1.5 L. of chloroform in divided portions, and the chloroform washed with 40 cc. of water. The dried, filtered solution was evaporated to a dark oil *in vacuo*. The oil was dissolved in 300 cc. of dry pyridine, treated with 75 cc. of acetic anhydride, and stored at 27° for sixteen hours. The solution was cooled in ice, excess acetic anhydride was decomposed by careful addition of 40 cc. of water, and 800 cc. of chloroform was added. Then 20% sulfuric acid was added in the cold until pH 2, and the layers were separated. The acid layer was extracted with 600 cc. of chloroform in divided portions and the combined chloroform extracts were washed with saturated sodium bicarbonate solution until basic, with water until neutral, and dried with sodium sulfate. Evaporation of the chloroform *in vacuo* gave a syrup (47 Gm.) which was chromatographed on an alumina column (2.2 x 37 cm.) in chloroform to give 33.5 Gm. (34%) of a yellow syrupy product. A sample was purified by molecular distillation at 0.1 mm., bath temperature 170°.

Anal.—Calcd. for $C_{19}H_{35}O_8PS_2$: C, 46.89; H, 7.25. Found: C, 47.41; H, 7.94.

Aldehydo-2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose.—To a stirred solution of 23.0 Gm. (0.0473 mole) of 2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose diethyl mercaptal and 44.5 Gm. of cadmium carbonate in 85 cc. of acetone and 30 cc. of water there was added, dropwise, a solution of 46.2 Gm. (0.17 mole) of mercuric chloride in 86 cc. of acetone during three hours. The mixture was stirred for 15 hours, and 5 Gm. of cadmium carbonate and 15 cc. of water was added After two hours of further stirring, the mixture was refluxed for one hour, the solids were filtered, and the filtrate was evaporated and azeotroped with benzene. The residue was extracted with 300 cc. of chloroform in divided portions and the filtered chloroform extracts were washed with 100 cc. of 40% potassium iodide solution and water. The dried, filtered solution was evaporated to leave 14.9 Gm. of dark syrup which could not be crystallized or distilled.

2 - (Methylthio) - 4,6 - diaminopyrimidine.—The product has been described (7) but the preparation was modified. To a solution of 7.55 Gm. (0.05 mole) of 2-thio-4,6-diaminopyrimidine hemihydrate (8) in 50 cc. of *N* sodium hydroxide and 25 cc. of water was added, slowly with shaking, 7.1 Gm. (0.05 mole) of methyl iodide. After one hour, the mixture was acidified to pH 5 with acetic acid and the product removed. It was recrystallized from hot water (Darco) and formed 4.95 Gm. (63%) of colorless needles, m. p. 185–186°. The reported (7) melting point is 185–186°.

6-Amino-4-[2',3',4'-tri-O-acetyl-5'-deoxy-5'-(diethyl phosphonate)-D-ribosamino]-2-(methylthio)-pyrimidine.—A solution of 17.5 Gm. (0.111 mole) of 2-(methylthio)-4,6-diaminopyrimidine, 10.5 Gm. (0.028 mole) of aldehydo-2,3,4-tri-O-acetyl-5-deoxy-5-(diethyl phosphonate)-D-ribose and 0.4 Gm. of ammonium chloride in 360 cc. of boiling absolute ethanol was allowed to stand at 28° for eighteen hours. The solvent was removed *in vacuo*, and the product dissolved in 50 cc. of chloroform. The filtered solution was poured onto an alumina column (1.5 x 25 cm.) and the column washed with fresh chloroform. The first 50-cc. fraction contained no product; the next 100 cc. was dark and gave a phosphorus-containing gum upon evaporation *in vacuo* weighing 7.2 Gm. Further fractions gave pure starting pyrimidine, which, when combined with the chloroform-insoluble residue weighed 12.38 Gm., so that 5.12 Gm. of the pyrimidine was consumed.

6-Amino-4-[2',3'-di-O-acetyl-5'-deoxy-5'-(diethyl phosphonate) - D - ribofuranosylamino] - 5 - (2'',5'' -

dichlorophenylazo) - 2 - (methylthio)pyrimidine.—A solution of 4.2 Gm. of crude 6-amino-4-[2',3',4'-tri-O-acetyl-5'-deoxy-5'-(diethyl phosphonate)-D-ribosamino]-2-methylthiopyrimidine in 100 cc. of methanol which had been saturated with ammonia at 0°, was allowed to stand for thirty-six hours at 25°. It was evaporated to dryness *in vacuo* and left a resin which was dissolved in 200 cc. of pyridine. The pyridine solution was poured into a stirred, ice cold neutral diazotized solution obtained from 1.6 Gm. of 2,5-dichloroaniline. The mixture was stirred for two hours at 0°, diluted with 600 cc. of water, cooled to 0°, and the product was filtered and dried (2.0 Gm.). The orange powder was soluble in ethyl acetate and pyridine. It was dissolved in 100 cc. of pyridine and chromatographed on alumina (column 1.5 x 40 cm.). Washing the column with pyridine gave phosphorus-free azopyrimidine while the ribosidic material remained adsorbed. The column was extruded, dried, and stirred for eighteen hours with 200 cc. of pyridine and 50 cc. of acetic anhydride. The excess anhydride was destroyed by adding ethanol to the cold solution and the mixture was filtered. The alumina was washed thoroughly with hot ethyl acetate and the combined filtrate and washings were evaporated *in vacuo*. The residue, a dark glass which did not crystallize, was heated with ethanolic picric acid and gave a picrate benzenate, yellow needles, m. p. 164–165° (softening at 160°) after recrystallization from benzene. Heating the product at 58° *in vacuo* in an attempt to remove the benzene resulted in loss of picric acid.

Anal.—Calcd. for $C_{30}H_{34}Cl_2N_9O_{15}P \cdot C_6H_6$: C, 44.45; H, 4.15. Found: C, 44.54; H, 3.45.

Attempts to diazotize 2,5-dichloroaniline according to Baddiley, *et al.* (9), resulted in an insoluble diazoaminobenzene derivative, a difficulty encountered by Noelting and Kopp in earlier work (10). The procedure of these authors gave much better results. A hot, rapidly stirred solution of 1.6 Gm. of 2,5-dichloroaniline in 4 cc. of concentrated sulfuric acid and 10 cc. of water was cooled rapidly and the fine crystal mass treated very slowly with a solution of 0.72 Gm. of sodium nitrite in 15 ml. of water at 5°. The filtered yellow solution was treated with a little urea and neutralized to pH 6 with pyridine.

5,6-Diamino-4-[2',3'-di-O-acetyl-5'-deoxy-5'-(diethyl phosphonate)-D-ribofuranosylamino]-2-(methylthio)pyrimidine.—A suspension of 120 Gm. of zinc dust and 15 Gm. of crude 6-amino-4-[2',3'-di-O-acetyl-5'-deoxy-5'-(diethyl phosphonate)-D-ribofuranosylamino]-5-(2",5"-dichlorophenylazo)-2-(methylthio)pyrimidine in 450 cc. of refluxing ethyl acetate was stirred rapidly while 60 cc. of glacial acetic acid in 600 cc. of ethyl acetate was added dropwise during one hour. The colorless mixture was filtered, the zinc washed three times with 200 cc. of boiling ethyl acetate, and the combined liquids were evaporated under nitrogen *in vacuo*. The residue was washed with petroleum ether and ether, and an alumina column (2.2 x 35 cm.) was prepared using ether. The petroleum ether and ether wash liquids were passed through and then the residue dissolved in 50 ml. of chloroform was poured on. The column was developed with ether, chloroform-ether 1:1, 1% ethanol in chloroform, and ethanol. The phosphorus containing material was eluted by chloroform. A portion was isolated as the yellow picrate by treatment with ethanolic picric acid and recrystallization from benzene, m. p. 140° (decompn.) (rapid heating).

9-[5'-Deoxy-5'-(diethyl phosphonate)-β-D-ribofuranosyl)]-6-amino-2-(methylthio)purine.—An ice-cold solution of 3 Gm. of chromatographed 5,6-diamino-4-[2',3'-di-O-acetyl-5'-deoxy-5'-(diethyl phosphonate)-D-ribofuranosylamino]-2-(methylthio)pyrimidine in 100 cc. of absolute alcohol was treated with sodium dithioformate prepared by substituting anhydrous, xylene-dried sodium sulfide in the method of Levi (11). This salt (2.4 Gm.) was dissolved in 15 cc. of alcohol and to it was added 0.6 cc. of acetic acid in 15 cc. of absolute alcohol. The mixture was allowed to stand for four hours at 0°, treated with 1.2 Gm. and 0.3 cc. of the same reagents, and stored overnight at 30°. After refluxing for one hour and evaporation of the solvent *in vacuo*, the residue was dissolved in 100 cc. of boiling ethyl acetate, filtered, evaporated *in vacuo*, dissolved in 30 ml. of benzene, decanted from a small residue, and poured onto 30 Gm. of alumina (column 2.2 cm. diameter). The column was washed with benzene until the washings were colorless, and the adsorbed dark band was eluted with 100 ml. of pyridine. Evaporation of the pyridine gave 500 mg. of a solid which was dissolved in 20 cc. of absolute methanol and treated with 1.5 ml. of *N* methanolic sodium methoxide solution. It was refluxed for four hours and stored for twelve hours at 30°. It was neutralized to pH 7 with glacial acetic acid and evaporated to a crystalline residue *in vacuo*. The residue was extracted with 100 cc. of boiling chloroform and the filtered extracts were evaporated *in vacuo* to leave a small amount of brown crystalline residue, soluble in hot water, alcohol, acetone, chloroform, and benzene, and insoluble in cold water, petroleum ether, and ether. It was washed with cold ether, and gave a poorly crystalline picrate. Treatment of the residue with platinic chloride in absolute ethanol gave the chloroplatinate as an amorphous powder which was washed with cold absolute alcohol, centrifuged, and freed of solvent by decantation and drying.

Anal.—Calcd. for $C_{15}H_{24}N_5O_6PS \cdot {}^1/_2H_2PtCl_6 \cdot 2H_2O$: C, 26.71; H, 4.33. Found: C, 26.68; H, 4.12.

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Studies on the Inhalation of Vapors from Radioactive Menthol and Camphor*

By ROBERT L. BOGNER and THOMAS C. GRUBB

Tritium "tagged" camphor and *l*-menthol were combined with other essential oils in a petrolatum base. Samples of bronchial air from dogs inhaling vapors of the "tagged" ointment in a closed system showed radioactivity after the second respiration. Air collected over an open container covered with the tagged ointment showed activity for a period of twelve hours' duration. Samples of bronchial air collected after the ointment was placed on the dog's chest showed activity up to twelve hours after a single application.

THE INHALATION of vapors from aromatic oils has been employed for centuries in the treatment of respiratory diseases. In recent years, volatile substances have also been inhaled for the treatment of sinusitis, bronchitis, asthma, laryngitis, etc , McClellan (1) and Currence (2). Since there is scant information available on the speed and duration of these inhaled vapors, it was decided to use radioactive campor and menthol as "tracers" for these determinations.

EXPERIMENTAL

Camphor and *l*-menthol were separately irradiated with tritium by the method of Wilzbach (3) Specific activities of 45 5 mc per Gm and 62 5 mc per Gm , respectively, were obtained These materials were added to other volatile substances (oil of nutmeg, oil of cedarleaf, oil of eucalyptus, and thymol) in a petrolatum base, thus providing a specific activity in the final mixture of 4 3 mc per 10 Gm of ointment

The dogs used in these experiments were prepared as follows: Under intravenous sodium pentobarbital anesthesia, the left chest wall was entered through the fourth intercostal space and a polyethylene tube was inserted into an opening made in the bronchus just below the bifurcation in two animals, and just below the second main division of the bronchus in three other dogs The tubes were sewed into position so that the samples of bronchial air from each respiration could be collected outside of the chest wall

In a typical experiment, 10 Gm of the radioactive ointment were spread over an area (8 cm x 10 cm) inside of a four-liter glass bottle which was partially submerged in a water bath maintained at 35 ± 0 5° (skin temperature) The dog was placed under pentobarbital anesthesia and its nose positioned into a mask connected to the bottle containing the tritiated ointment Suitable valves and tubing were provided so that at each inhalation, air was drawn over the ointment and into the dog's respiratory tract, as shown in Fig. 1 Samples of expired air were collected by placing the tubing from the dog's bronchus inside a small glass bottle filled with water and submerged in a shallow tray of water. About 5 cc of air were collected at each expiration Separate sample bottles were filled with each expiration The bottles were capped under water and taken to the counting laboratory, where the radioactivity of their contents was determined as follows: A two-liter counting chamber was evacuated to about 10 μ and the sample allowed to expand into the counting chamber, which was then filled to atmospheric pressure with methane The counting chamber was placed inside a ring of anticoincidence counters surrounded by a steel shield, 20 5 cm thick The sample was then counted on the plateau in the proportional counting region The normal background count was 60 c p m (counts per minute) Samples of the expired air were collected after the 2nd, 4, 5, 6, 7, 10, 13, 15, and 19th respirations following the initial inhalation of the vapor-containing air Thus, air samples were obtained as early as four seconds and as late as eighty seconds after the initial inspiration of the "tagged" vapors

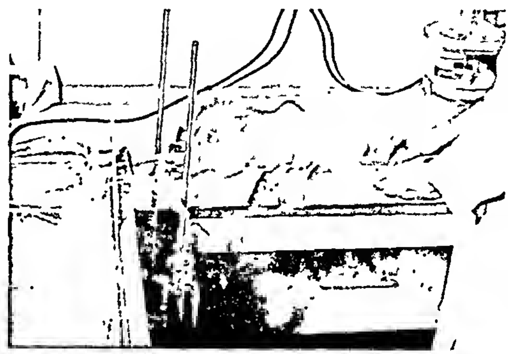

Fig 1—This photograph shows the glass bottle containing the "tagged" ointment immersed in a water bath The vapors are inhaled through the mask over the dog's nose and the inhaled air is collected from the tube coming from the dog's chest The investigator is collecting the air samples under water

The results indicated that irradiated vapors were detectable after the second respiration (five and eight seconds) in two of the dogs Samples from the other dogs contained "tagged" vapors after the 5th, 7th, and 10th respirations The specific activity of these samples ranged from 100 net c p m. per cc of air after five seconds contact to 1,000 c. p. m. per cc. of air in the sample taken thirty-three seconds after the initial inhalation

In order to determine the duration of therapeutic vapors released from the ointment application area, an *in vitro* and an *in vivo* experiment were carried out as follows:

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Fig. 2.—This photograph indicates the position of the dog's nose in relation to the "tagged" ointment rubbed on his chest. Note the indwelling tube, coming from the intercostal space, to which a syringe is attached to collect the air sample.

In the *in vitro* experiment, 10 Gm. of the "tagged" ointment having an activity of 11 mc. per Gm., were spread over the bottom of a stainless steel pan (15 x 25 x 5 cm.) resting in a water bath maintained at 35 ± 0.5°. A glass funnel 5 cm. in diameter was inverted 2.5 cm above the surface of the ointment. At various time intervals, 25 cc. of air were drawn into a glass syringe connected to the stem of the funnel by a 5-cm. length of Tygon tubing These samples were assayed for tritium activity, according to the method described. Samples of air were taken after 1 minute, 30 minutes, and one, three, five, eight, and twelve hours, and the activity reported for each of these samples, respectively, was as follows: 3,880, 1,393, 750, 120, 93, 177, and 90 net c. p m per cc. of air.

In the *in vivo* experiment, a dog prepared with an indwelling bronchial cannula just below the bifurcation, as described, was treated as follows: Following anesthesia of the animal with sodium pantobarbital, 10 Gm. of the radioactive ointment were spread over an area 7 5 x 7 5 cm. on the shaved chest and covered with a light piece of gauze The dog's nose was positioned 7 5 cm from the inuncted area as shown in Fig. 2. Samples of air were withdrawn by aspiration from the cannula into a 30-cc glass syringe at the rate of 5 cc per respiration until a 25-cc. sample was collected in the syringe. This experiment was conducted out-of-doors with a temperature of 21–27°, and the samples collected after eight and twelve hours' exposure to the vapors were found to have a specific activity of 958 and 647 net c. p. m per cc of air, respectively.

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Catalytic Hydrogenation of Derivatives of α-Cyano-β-(1-naphthyl)acrylic Acid*

By JAMES E. GEARIEN, MASUMI NAKAMICHI, and KENNETH J. LISKA†

The ethyl ester and amide of α-aminomethyl-β-(1-naphthyl)propionic acid were prepared by the catalytic hydrogenation of the corresponding cyanonaphthylacrylic acid derivative. These compounds may be regarded as analogs of lysergic acid derivatives.

DERIVATIVES of α aminomethyl-β-(1-naphthyl) propionic acid (II) possess molecular structures that might be considered analogous to rings B, C, and D of the lysergic acid molecule (III) and, therefore, might possess the pharmacological activity of the ergot alkaloids. The amide and ethyl ester of α-aminomethyl-β-(1-naphthyl)-propionic acid were prepared by the catalytic hydrogenation of the amide and ethyl ester of α-cyano-β-(1-naphthyl)acrylic acid (I).

Ethyl α-cyano-β-(1-naphthyl)acrylate has been made by the condensation of the sodium salt of cyanoacetic acid with 1-naphthaldehyde to yield the sodium salt of α-cyano-β-(1-naphthyl)-acrylic acid which was then esterified (1). A single step synthesis, reported by Fulton and Robinson (2), utilized the condensation of ethyl cyanoacetate with 1-naphthaldehyde. By using morpholine as the catalyst instead of the recommended piperidine and by removing the water as it is formed during the reaction, the yield of ethyl α-cyano-β-(1-naphthyl)acrylate was increased from 48 to 75 per cent of the theoretical.

The previously unreported α-cyano-β-(1-naphthyl)acrylamide was synthesized by the condensation of cyanoacetamide with 1-naphthaldehyde. Maximum yields were obtained when morpholine was used as the catalyst and when the reaction was carried out without a solvent.

Hydrogenations of the ester and amide of α-cyano-β-(1-naphthyl)acrylic acid were carried out at room temperature and low pressures using W-1 Raney nickel as the catalyst. These

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An abstract of part of a dissertation submitted by Kenneth J Liska to the faculty of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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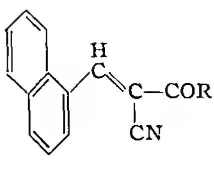
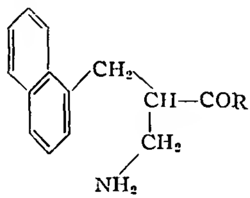
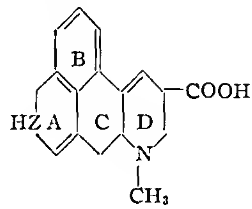


I
R=NH_2, OC_2H_5



II
R=NH_2, OC_2H_5



III

reactions were successful only when a large ratio of catalyst to hydrogen acceptor was employed and when ammonia was added to the hydrogenation mixture to reduce the formation of secondary amines (3, 4) Successful hydrogenation of α-cyano-β-(1-naphthyl)acrylamide was possible only when the weight ratio of catalyst to hydrogen acceptor was as great as two to one. Under these conditions and in the presence of small amounts of ammonia, crude yields were as high as 77 per cent of the theoretical, but the removal of impurities resulted in the loss of considerable amounts of the product Hydrogenation of ethyl α-cyano β-(1-naphthyl)acrylate proceeded smoothly with a one to one ratio of catalyst to hydrogen acceptor to give a 51 per cent yield of the expected product. Without the addition of ammonia, the yield was only half of that reported. The amount of ammonia, however, could not be further increased without reducing the yield of desired product due to ammonolysis

The ultraviolet absorption curves of the two α-aminomethyl-β-(1-naphthyl)propionic acid derivatives in 95 per cent ethanol exhibited three maxima in the region 270–295 mμ, indicative of an intact naphthalene system with no exocyclic conjugation (5)

In a preliminary pharmacological screening, the compounds did not exhibit pharmacological activity.[1]

EXPERIMENTAL[2]

Ethyl α-Cyano-β-(1-naphthyl)acrylate.—A mixture of 15 6 Gm (0 1 mole) 1-naphthaldehyde, 11 3 Gm (0 1 mole) ethyl cyanoacetate, one ml of morpholine, and 75 ml of dry benzene was heated at reflux temperature in a Dean-Stark moisture determination apparatus until the water collected came to a constant volume After the benzene was removed by distillation under reduced pressure, the residual oil solidified on cooling The solid was recrystallized from 80 ml. of boiling ethanol using charcoal for decolorization. This gave 18 5 Gm (74%) of yellow needles, m p 81 5–82° Hopkins and Chisholm (1) reported 81–82°.

α-Cyano-β-(1-naphthyl)acrylamide.—A mixture of 15 6 Gm (0 1 mole) of 1-naphthaldehyde and 8 4 Gm (0 1 mole) of cyanoacetamide was heated on the steam bath for five minutes Morpholine (1 ml) was quickly added, the reaction flask was fitted with a reflux condenser and agitated briskly Heating was resumed and in a few minutes the contents of the flask solidified After an additional ten minutes of heating, the flask was cooled to room temperature, its contents ground up in a mortar, and the solids treated with 20 ml of ethanol to remove most of the color One recrystallization from a large volume of ethanol then gave 16 6 Gm (74%) of cream-colored needles, m p 194–195° After a second recrystallization from absolute ethanol-acetone (1 1), the analytically pure sample melted at 195 5–196°λ max (95% ethanol): 257, 330 (broad) mμ

Anal —Calcd for $C_{14}H_{10}N_2O$: N, 12 61 Found: N, 12 66

Ethyl α-Aminomethyl-β-(1-naphthyl)propionate.—A 10 1-Gm (0 04 mole) sample of finely powdered ethyl α-cyano-β-(1-naphthyl)acrylate was suspended in 210 ml of absolute ethanol To this suspension was added 11 6 Gm of freshly prepared W-1 Raney nickel (6) and 10 4 ml of ethanolic ammonia solution containing 1 4 Gm (0 08 mole) of ammonia The mixture was hydrogenated for three and one-half hours at an initial pressure of 46 p s i The contents of the pressure bottle were filtered free of catalyst, and the filtrate evaporated A light yellow oil remained It was distilled under reduced pressure, b p 132–138° (0 11 mm) The yield of pale yellow oil was 5 2 Gm (51%) λ max (95% ethanol)· 272 5, 282 5, 291 mμ

Anal —Calcd for $C_{16}H_{19}NO_2$ C, 74 68; H, 7.44; N, 5 44 Found: C, 74 77; H, 7 46; N, 5 25.

α-Aminomethyl-β-(1-naphthyl)propionamide.—To a suspension of α-cyano-β-(1-naphthyl)acrylamide (4 4 Gm , 0 02 mole) in 220 ml of absolute ethanol was added 8 0 Gm of W-1 Raney nickel and 18 5 ml of an ethanolic ammonia solution which contained 3 4 Gm (0 02 mole) of ammonia The reactants were shaken under an initial hydrogen pressure of 46 p s i for four hours After the catalyst was removed, the solvent was evaporated, and the residue, which had solidified to a hard mass when cooled to room temperature, was broken up and triturated with 15 ml of anhydrous ether. A tan powder resulted (3 52 Gm , crude yield) which was recrystallized from a small volume of chloroform to give 1 9 Gm (42%) of white crystals, m p 157–158°. For analysis, the compound was further purified by suspending the 1 9 Gm. in 25 ml of water, adding 25 ml of concentrated hydrochloric

[1] The authors are indebted to Abbott Laboratories for subjecting the compounds to their pharmacological screening procedures

[2] Melting points are not corrected Analyses were performed by Weiler and Strauss Analytical Laboratories, Oxford, England

acid, filtering the solution, and then making alkaline with 20% sodium hydroxide solution The white masses so obtained were again recrystallized from chloroform, m p 157–158 °

Anal—Calcd for $C_{14}H_{16}N_2O$: C, 73 65, H, 7 07; N, 12 27 Found: C, 73 41; H, 7 22, N, 12 12

The hydrochloride salt of α-aminomethyl-β-(1-naphthyl)propionamide was prepared by dissolving the compound in a small volume of absolute ethanol and adding an excess of anhydrous ether saturated with hydrogen chloride gas The hydrochloride was recrystallized by dissolving it in the smallest possible volume of absolute ethanol, chilling the solution, and adding an excess of anhydrous ether White crystals which melted at 243–244° were obtained

Anal—Calcd for $C_{14}H_{17}ClN_2O$· N, 10 58; Cl, 13 39. Found. N, 10.78; Cl, 13 29

SUMMARY

1. The ethyl ester and the amide of α-aminomethyl-β-(1-naphthyl)propionic acid have been prepared as potential oxytocic agents.

2 The intermediates ethyl α-cyano-β-(1-naphthyl)acrylate and α-cyano-β-(1-naphthyl)acrylamide have been prepared the former by a new procedure.

3 When subjected to pharmacological screening, the compounds failed to exhibit significant activity.

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The Influence of Gibberellic Acid on the Growth and Alkaloid Biogenesis in *Datura stramonium* Linné and in *Atropa belladonna* Linné*

By GORDON M. SMITH and LEO A. SCIUCHETTI

Gibberellic acid in concentrations of 100 and 1,000 p. p. m. was administered as an aqueous spray to the leaves and tops of *Datura stramonium* and *Atropa belladonna*. Characteristic effects on the growth of the treated plants are described. Significant increases in plant height were demonstrated. The fifty-four-day-old stramonium plants were harvested four weeks after the first treatment. Fresh and dry weight data indicated increases in total, leaves and tops, and stem weights, but decreased root weight. The twelve-week-old belladonna plants were harvested seven weeks following treatment. Appreciable decreases in leaves and tops and root dry weights were noted. However, this was accompanied by significant increases in stem weight. Stramonium plants receiving both a single and double spray treatment with each concentration of gibberellic acid showed decreases in the concentration of total alkaloids in the leaves and tops and in the stems. Increased alkaloid production was observed in the roots. The favorable effects on growth from the two-spray treatment of 100 p. p. m. of gibberellic acid resulted in significant increases in total alkaloid production. Significant decreases in the concentration of alkaloids in the plant organs of belladonna were noted. Further, the total alkaloid content of belladonna treated with gibberellic acid was about one-half that of the untreated plants.

NUMEROUS PAPERS have recently appeared reporting the various effects of gibberellic acid or the gibberellins on plant growth (1–7) Further, specific effects have been demonstrated in members of the *Solanaceae*. For example, Lang (8) has shown gibberellic acid effectively substitutes for the cold requirement of biennial *Hyoscyamus* Also, evidence has been presented to indicate that an increase in cell division resulted from the application of gibberellins to biennial *Hyoscyamus niger* (9). However, the available literature has not indicated that any studies have been performed on the effect of this growth-inducing chemical on alkaloid biogenesis in the *Solanaceae* The purpose of this study was to investigate, under controlled greenhouse conditions, the influence of gibberellic acid on the growth and alkaloid biogenesis in *Datura stramonium*. It was further decided to extend this line of investigation to a closely related plant, *Atropa belladonna*. With the latter it was decided

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The study with belladonna was done by L A Sciuchetti and supported in part by funds from General Research of the Graduate School of Oregon State College

to change the frequency of application of gibberellic acid and the environmental conditions of growth, *viz.*, growing the plants under field conditions.

EXPERIMENTAL

Procedure.—*Datura stramonium* plants used in this study were grown under carefully controlled greenhouse conditions Seeds were germinated in culture flats containing soil composed of equal parts of sand, peat moss, and sterilized soil Sixty-two sixteen-day-old seedlings were transplanted into 2 x 2-inch paper bands in flats containing soil composed of two parts sandy loam and one part peat moss. On July 15, 1957, the twenty-two-day-old seedlings which had attained a height of approximately 5.5 cm. were transplanted into individual clay pots (8 x 8 inches) On July 19, 1957, 60 of the potted plants were selected for this study and divided into three groups of 20 plants each. The first group constituted the control or untreated plants; the second were those treated with 100 p p. m. of gibberellic acid (G. A);[1] and the third were those treated with 1,000 p. p. m of G A Freshly prepared aqueous solutions of G A. were administered in the form of a spray to the leaves and tops of the treated plants. At the start of the third week, half of the plants in each of the treated groups received a second spray treatment with the aforementioned concentrations of G. A. The fifty-four-day-old plants were harvested four weeks after the first treatment.

The belladonna seeds were germinated in a manner similar to that for stramonium. On July 15, 1957, 100 two-week-old seedlings were transplanted into 2 x 2-inch paper bands in flats containing soil composed of two parts of sandy loam and one part of peat moss. On July 19, 1957, 84 of the banded plants were selected for this investigation and divided into three groups of 28 plants each. The plan for treating each group was similar to that for stramonium. The first treatment was administered to groups 2 and 3 at this time.

Ten days later the treated plants were given a second application of G. A. At this time the plants were transplanted into a prepared plot of ground in the drug garden north of the pharmacy building. The area chosen for this study was at the perimeter of a plot of ground in which digitalis plants had been planted. The light intensity in this area varied considerably due to the shade from the building and surrounding trees. The belladonna plants were spaced 2 feet apart in double rows Each hill into which the belladonna was transplanted was fertilized by thoroughly working into the soil 15 Gm. of lime (calcium carbonate) and 20 Gm. of complete organic fertilizer.[2] Previous analysis of soil samples from this plot indicated that fertilization was desirable.

A third treatment with G. A. was given ten days later to the plants in groups 2 and 3. Ten plants were picked at random from each group and were harvested. This was seven weeks after the final treatment. The remaining plants were allowed to remain for further study.

During the harvest period each species was divided into three portions; leaves (with flowering and fruiting tops), stems, and roots. Fresh weights were promptly taken of each individual portion and the fresh material was immediately transferred to a hot-air circulating drier. The leaves and root portions were dried at a temperature of 48.5° for thirty hours while the stems were dried forty hours Upon removal from the drier the plant parts were placed in a desiccator, allowed to attain room temperature, and then weighed. The portions were placed in airtight metal containers until subsequent pulverization into a No. 40 powder in a Wiley mill. The powdered material was then stored in airtight colored-glass containers until subsequent analyses for alkaloids were performed.

Growth Effects in Stramonium.—The plants were carefully observed daily for specific growth effects during the four-week treatment period. The height of each plant was taken at approximately the same time each day and was determined by measuring the distance in cm. from the base of the stem to the active growing tip of the plant. In general, after two weeks, the plants treated with G. A. demonstrated the following differences compared with the controls: increased height due primarily to increased stem elongation; leaves which were longer, thinner and slightly chlorotic; less succulent growth; increased rate of flowering and plant maturity. Similar effects have previously been reported in other plants (1–6, 11). At the end of the fourth week the plants receiving a two-spray treatment demonstrated the "gibberellin" effects previously indicated, but to a greater extent (Fig. 1). However, at this time, the plants receiving only one treatment with G. A. appeared to resemble more closely controls in growth characteristics with the exception that the leaves remained slightly chlorotic. It was further observed that plants treated with G. A. were more prone to insect attacks. This occurred in spite of the fact that all plants were

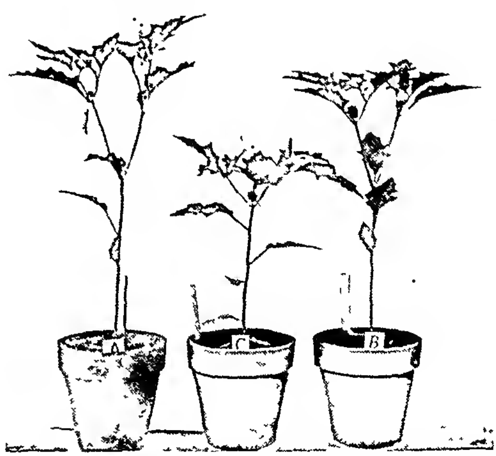

Fig. 1.—Effect in *Datura stramonium* after four weeks from a two-spray treatment with gibberellic acid. A = 1,000 p. p. m., B = 100 p. p. m., C = control.

[1] The gibberellic acid used in this study was supplied through the courtesy of Dr Curt Leben, Eli Lilly & Co, Indianapolis, Ind.

[2] Organic Morcrop, Chas Lilly Co., Seattle, Wash. (Analysis—5% of total nitrogen, 3% available phosphate, 2% available potash.)

dusted twice weekly with Maladusto 4D[3] (4% malathion dust).

The most characteristic effect induced in stramonium by the treatment with G. A. was increased stem elongation due mainly to an increase in length between the internodes of the stem (Fig. 1). This resulted in considerable increases in the height of the treated plants (Fig. 2). Compared with the controls, the plants treated with both concentrations of G. A. displayed increases in height of about 45% at the first week and approximately 36% at the second week. The group treated with a single spray of 100 p. p. m. of G. A. indicated increases in height of approximately 25% and 12% at the third and fourth weeks, respectively. Correspondingly, the plants treated with the higher concentration of G. A. (1,000 p. p. m.) showed increases of 26% and 13%, respectively. Significant increases in height were induced in those plants receiving a second spray of G. A. For example, at the lower concentration the increases were 56% and 35% at the third and fourth weeks, while at the higher concentration the corresponding increases were 51% and 49% (Fig. 2).

Growth Effects in Belladonna.—The belladonna plants treated with G. A. were somewhat taller than the controls at the time of transplantation (ten days after the first treatment). After the second treatment the treated plants grew more rapidly than the controls Further, at this time it was observed that the plants treated with G. A. were more prone to insect attacks than the untreated, even though all plants in the drug garden were dusted twice weekly with 4% malathion. This characteristic was noted thereafter throughout the growth period. The growth effect from G. A. was much more pronounced after the third treatment. This was characterized by an immense elongation between the stem internodes and the formation of numerous lateral shoots at the internodes. Gray (6) has reported a similar effect in bean plants. The plants were much more spindly than the untreated and did not appear as healthy. From the fourth week following treatment until the plants were harvested the following growth characteristics were observed in the treated plants: they were spindly and vine-like; the leaves were slightly chlorotic, narrower, thinner, and less succulent; some of the younger leaves were crinkled and appeared to be blistered; the plants were taller; the onset of flowering was more rapid; and, in general, they did not appear as healthy as the controls (Fig. 3). In general, many of the gibberellin effects were similar to those displayed in *Datura stramonium.* Also Gray (6) has reported that the leaves of pepper plants became rough instead of smooth and tobacco leaf blades became more elongated, following a spray treatment with G. A.

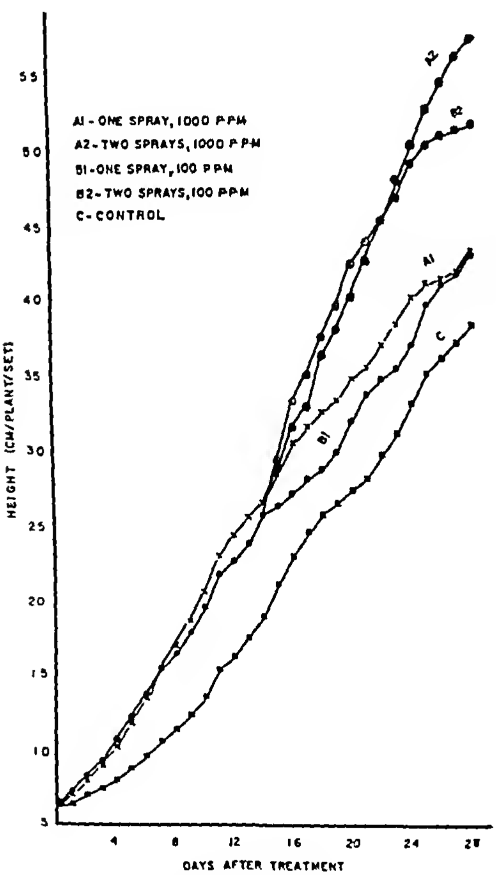

Fig. 2.—Increase in height of *Datura stramonium* by treatment with gibberellic acid.

Fig. 3.—Growth effects in *Atropa belladonna* seven weeks after treatment with gibberellic acid. A = 1,000 p. p. m., B = 100 p. p. m., C = control.

The most pronounced effect induced by the G. A was increased stem elongation due primarily to an increase in the length between the internodes of the stems (Fig. 3). Significant increases in the height of the treated plants were observed from measurements taken at the fourth, sixth, and seventh week following the last treatment (Fig. 4). The plants treated with 100 p. p. m. of G. A. attained a height of approximately 312%, 224%, and 191% of the controls at the fourth, sixth, and seventh week, respectively. The corresponding figures for the plants treated with 1,000 p. p. m. of G. A. were 370%, 271%, and 241% (Fig. 4). The plants treated with the higher concentration of G. A. displayed the greatest increases. Phinney (2) has reported that the degree of response from gibberellin in corn seedlings was related to dosage, and Marth, *et al.* (4), have shown that many plants were 50–300% taller within three to four weeks following treatment with G. A.

Correlation with Fresh and Dry Weight Data.—In stramonium, significant increases in total weight were demonstrated from all the treatments, ranging

[3] Manufactured by Miller Products Co., Portland, Ore

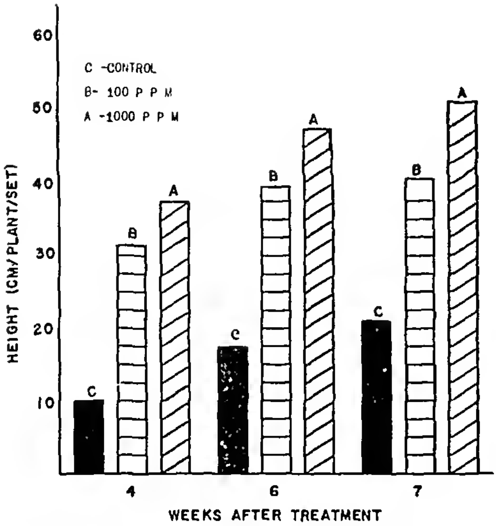

Fig 4 —Height of *Atropa balladonna* after treatment with gibberellic acid

from 120.5 to 159.7% of the controls (Table I) The plants receiving two treatments of 100 p p m of G A displayed the largest increase (56.6%) in the leaf and top weights, while those receiving two sprays at the higher concentration displayed the smallest increase (14.1%) Significant increases in stem dry weights were shown from all the treatments The stramonium plants treated with two sprays of G A demonstrated the largest increases The largest increase (91.3%) was shown with the lower concentration of G A (Table I) On the other hand, the group receiving two treatments of the higher concentration indicated the greatest stem growth when leaf/stem ratios were employed Decreases in root dry weights were demonstrated from all the treatments The largest decrease (31%) was indicated by the plants receiving two treatments of the higher concentration The inhibition of root growth by the G A was further demonstrated when comparing shoot/root ratios which were calculated from dry weight data

With belladonna, significant decreases in leaves and tops and root dry weights were indicated from both treatments (Table II) The decreases were greater in the plants treated with the lower concentration of G A However, this was accompanied by significant increases in stem dry weights The greatest increase was displayed by plants treated with the higher concentration of G A No significant changes in total dry weights were indicated The leaf/stem ratio for the control group was 5.09 and that for the plants treated with the lower and higher concentrations of G A were 1.66 and 1.53 This further demonstrates the profound effect that G A had on stem growth Inhibition of root growth was further indicated from the G A since the shoot/root ratios for both treatments were greater than the controls

In both plants, when the dry weights of the shoot organs were divided by their fresh weights, the controls indicated the greatest loss of moisture This confirms the observations made during the growth period that control shoots appeared more

TABLE I —WEIGHTS OF STRAMONIUM PLANT PARTS (AV /PLANT/GROUP)

Treatment	No of Plants	Total Weight Fresh Gm	Total Weight Dry Gm	Total Weight Dry/Fresh %	Total Weight Control Dry Wt %	Leaves and Tops Fresh Gm	Leaves and Tops Dry Gm	Leaves and Tops Dry/Fresh %	Leaves and Tops Control Dry Wt %	Stems Fresh Gm	Stems Dry Gm	Stems Dry/Fresh %	Stems Control Dry Wt %	Roots Fresh Gm	Roots Dry Gm	Roots Dry/Fresh %	Roots Control Dry Wt %
Control	10	29.8 ± 1.8	3.95 ± 0.2	13.3		14.7	1.98	13.4		9.9	1.38	13.9		5.2	0.58	11.2	
100 p p m G A, One spray	8	31.0 ± 1.7	4.76 ± 0.3	15.4	120.5	15.9	2.52	15.8	127.3	10.5	1.77	16.9	128.3	4.7	0.47	10.0	81.0
100 p p m G A, Two sprays	8	41.6 ± 0.9	6.31 ± 0.2	15.2	159.7	20.8	3.10	14.9	153.6	15.8	2.64	16.7	191.3	5.0	0.56	11.2	96.6
1,000 p p m G A, One spray	8	34.5 ± 0.9	4.91 ± 0.4	14.2	124.3	17.6	2.55	14.5	128.8	12.7	1.90	15.0	137.7	4.3	0.46	10.7	79.3
1,000 p p m G A, Two sprays	8	35.0 ± 2.6	4.99 ± 0.4	14.3	126.3	16.2	2.26	14.0	114.1	16.1	2.33	14.5	164.5	2.7	0.40	14.8	69.0

TABLE II.—WEIGHTS OF BELLADONNA PLANT PARTS (AV./PLANT/GROUP)

	Total Weight			Leaves and Tops			Stems			Roots		
Treatment	Fresh, Gm	Dry, Gm	Control Dry Wt., %	Fresh, Gm	Dry, Gm	Control Dry Wt., %	Fresh, Gm	Dry, Gm	Control Dry Wt., %	Fresh, Gm	Dry, Gm	Control Dry Wt., %
Control	52.86	4.90	.	38.77	3.31	.	7.49	0.65		6.60	0.94	
100 p. p. m. G. A	40.81	4.45	90.8	23.84	2.39	72.2	12.22	1.44	221.5	4.75	0.62	66.0
1,000 p. p. m. G. A.	45.23	4.96	101.2	25.36	2.59	78.3	14.98	1.69	260.0	4.89	0.68	72.3

succulent. This suggests that more carbohydrates and/or proteins may have accumulated in the shoot organs of the treated plants.

Analysis for Alkaloids.—The dried plant parts, using pooled samples, were assayed for total alkaloids according to the Witt-Youngken method (10), substituting chloroform for benzene as the immiscible solvent. In stramonium, considerable decreases in the percentage of total alkaloids (expressed as scopolamine) were generally indicated in the leaves and tops and in the stems, while increases were shown in the root organs. The concentration of alkaloids in the leaves and tops decreased 30.3 and 27.4%, respectively, for plants receiving the single spray treatment of the higher and lower concentrations of G. A. These decreases were considered significant. Significant decreases, ranging from 21.7 to 48.3%, were found in the accumulation of alkaloids in the stems. The greatest decrease was exhibited by the group treated with a single spray of 100 p. p. m. of G. A. Considerable increases in total alkaloid production in the roots were shown from the treatments. The increases due to the two-spray treatments ranged from 41.7 to 50% and were considered significant. The group receiving a two-spray treatment of 100 p. p. m. of G. A. displayed the highest concentration of alkaloids in the roots, *viz.* 150% of the controls.

In belladonna, decreases in the concentration of alkaloids of all the organs were demonstrated from both treatments. For example, belladonna treated with 100 p. p. m. of G. A. contained approximately 57, 53, and 84% of the total alkaloids of the controls in the leaves and tops, stems, and roots, respectively. The corresponding figures for the plants treated with 1,000 p. p. m. of G. A. were about 55, 43, and 77%.

Total Plant Alkaloids.—It was further desired to make comparisons based on the total alkaloids per plant and per plant organ. The total alkaloids (calculated as scopolamine) were obtained by multiplying the dry weight of the plant organ by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table III). The most favorable response on total alkaloid production in stramonium was induced by the two-spray treatment with 100 p. p. m. of G. A. This treatment caused a decrease in the concentration of alkaloids in the shoot organs. However, the favorable response on growth resulted in a 46% increase in total plant alkaloids.

Significant decreases in total alkaloids (calculated as hyoscyamine) were found in the belladonna plant, leaves and tops, and roots, whereas increases were observed in the stems (Table IV). In general, the total alkaloid content of the treated plants was about one-half that of the controls.

TABLE III.—TOTAL ALKALOID CONTENT OF *Datura stramonium*[a]

	Per Plant		Leaves and Tops		Stems		Roots	
Treatment	Alkaloids, mg	Control, %	Alkaloids, mg	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %
Control	9.91		6.83		2.80		0.28	.
100 p. p. m. G. A., One spray	8.52	86.0	6.40	93.7	1.86	66.4	0.26	92.9
100 p. p. m. G. A., Two sprays	14.46	146.0	9.86	144.5	4.20	150.0	0.40	142.9
1,000 p. p. m. G. A., One spray	8.70	87.8	6.22	91.1	2.22	79.3	0.26	92.9
1,000 p. p. m. G. A., Two sprays	10.75	108.6	6.78	99.3	3.70	132.1	0.27	96.4

[a] Alkaloid content for plant parts calculated from dry wt and alkaloid analysis data, total plant alkaloid = leaves and tops + stems + roots All data were based on av /plant/group

TABLE IV.—TOTAL ALKALOID CONTENT OF BELLADONNA[a]

	Per Plant		Leaves and Tops		Stems		Roots	
Treatment	Alkaloids, mg	Control, %	Alkaloids, mg	Control, %	Alkaloids, mg	Control, %	Alkaloids, mg.	Control, %
Control	19.05		12.25		2.76		4.04	...
100 p. p. m. G A	10.29	54.0	4.59	37.5	3.25	117.8	2.45	60.6
1,000 p. p. m. G A	10.05	52.8	4.88	39.8	3.11	112.7	2.06	51.0

[a] Alkaloid content for plant parts calculated from dry wt and alkaloid analysis data, total plant alkaloid = leaves and tops + stems + roots Based on av /plant/group

DISCUSSION

Characteristic responses were induced in *Datura stramonium* and *Atropa belladonna* by the spray treatments with G. A. Some of these responses have been reported in *Hyoscyamus niger* (8), pepper, tomato, and tobacco (6), and in various other plants (11). The most profound effect was the increased height of the treated plants due to increased stem elongation. Belladonna responded to this gibberellin effect to a greater extent than did stramonium. Further, it was observed that plants treated with G. A. were more prone to insect attack and did not appear as healthy and sturdy as the untreated plants. With belladonna, the treated plants required more labor in tying the plants to stakes in order to keep them upright. This was necessary because of the greatly increased number of axillary shoots and the vine-like and spreading-type of plant growth. These factors would present serious obstacles to the commercial cultivation of belladonna.

Different results between the two species regarding dry weights were induced by the treatments. In stramonium, significant increases in shoot dry weights were generally observed, accompanied by decreases in root dry weights. In belladonna, significant reductions in the dry weights of the leaves and tops and the roots were offset by significant increases in the dry weights of the stems. This resulted in slight but insignificant changes in the total dry weight of the treated plants. Inhibition of root growth was demonstrated for both species as indicated by reduced weights and increased shoot/root ratios. In the case of belladonna, this factor accompanied by no significant increase in total dry weight poses a serious limitation for the practical use of G. A. under conditions similar to this experiment.

Of more importance from a commercial aspect was the profound effect that G. A. had on alkaloid formation and accumulation in the plants. It was found in stramonium that a two-spray treatment with 100 p. p. m. of G. A. resulted in a 46% increase in total plant alkaloids. Increasing the concentration of G. A. was less beneficial, while the one-spray treatment of both concentrations proved to be unfavorable. On the other hand, treated belladonna plants contained about one-half the total amount of alkaloids as did the controls. These inconsistencies could be attributed to generic differences. However, it would appear that an optimum condition involving concentration and frequency of application is necessary for maximum beneficial effects. This optimum condition is now being investigated. Further, observations made during the growth of both species and from a study of the data suggest that changes in the carbohydrate and/or protein metabolism were induced by G. A. Research is now being directed toward a study of this possibility.

It was noted generally that in both species the concentration of G. A. was not critical to certain effects. There were some differences due to the strength of G. A. employed on increased height, fresh and dry weights, alkaloid concentration, and total alkaloid content. However, these differences were not considered significant. The unfavorable effect of G. A. on the alkaloid production in belladonna indicates that the concentrations of G. A. employed in this study were toxic. It is altogether possible that favorable responses in both growth and alkaloid production may have been induced with lower concentrations of G. A.

From this study the following similarities were observed in both species: the treated plants indicated significant increases in stem growth and inhibition of root growth; the treated plants appeared to lack natural resistance to insect attack; and, the shoots of the treated plants were shown (by observation and other data) to be less succulent than untreated plants. On the other hand, some differences were noted between the two species. The increases in height and stem growth were much greater in belladonna and the decreases in the alkaloid content of this species were greater. It should be pointed out that both plants were treated with similar concentrations of G. A. by spraying onto the leaves and tops. On the other hand, the following factors were different; the environmental conditions, the frequency of treatments, and time interval between the following treatments, the age of the plants. Of these factors, it would appear that the number of treatments and the time interval between and following treatments would be important considerations. The observations that the treated plants had decreased resistance to insects, were less succulent, and had less root growth suggest the possibility that essential nutrients required for normal plant metabolism and growth were being used for the increased stem growth. The decreased alkaloid content of *Atropa belladonna* and, in some cases, of *Datura stramonium*, further suggests that some of the nitrogenous material normally used for the biosynthesis of alkaloids was diverted toward increased stem growth and other metabolic pathways.

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Antibacterial Activity of the Heartwood of *Haematoxylon braziletto**

By ROBERTSON PRATT and YOKO YUZURIHA

Aqueous extracts of the heartwood of the Mexican plant, *Haematoxylon braziletto*, contain a substance which, in appropriate concentrations, is bactericidal for *Salmonella typhosa* and *Micrococcus pyogenes* var. *aureus* and is bacteriostatic for *Escherichia coli*. The active principle has not been unequivocally identified but it is suggested that the antibacterial action is due to the pigment, brazilein, or to its precursor, brazilin, or to some derivative(s) of these. Neither the unpigmented sapwood nor the bark of the plant contains the active principle. Hematoxylin, which is derived from the heartwood of the related plant, *H. campechianum*, and which contains pigments that are closely related chemically to those of *H. braziletto*, has similar antibacterial activity in similar concentrations.

The history of pharmacy and medicine is replete with the wisdom as well as the folly of folklore, and a number of our most respectable drugs can be traced to very early beginnings in the empirical treatment of infectious, functional, or mental illnesses. Antibiotics, hormones, and tranquilizers are but three examples of classes of recently rediscovered drugs which, in the form of crude preparations, have been used therapeutically for centuries.

Therefore, our curiosity was aroused by receipt of a letter referring to *Haematoxylon braziletto* as follows:[1]

"... While doing field work in the Gulf of California and later on the mainland of Sonora, I found the people there using this plant for medicinal purposes. A sliver of wood dropped into a glass of water very quickly produces a pink solution strongly resembling permanganate of potash. The potion is then drunk as a remedy for "whatever ails you." I also found them placing this wood in drinking troughs of their chickens and, in some districts, in the water of the rural schools.

"... My question is, then, have the natives employed a drug for medicinal purposes where we have remained unaware of such value and used it merely in microtechnics?"

H. braziletto is limited in distribution to Lower California and adjacent states on the mainland of Mexico where, according to Gentry (1), it is common on mesas and arid hilltop slopes at elevations from 800 to 2,500 feet in Sonora and Chihuahua. At lower elevations it attains the size of a small tree but at higher levels (about 1,800 feet) it forms "low dense, hemispherical shrubs" occasionally so dense as to form "impenetrable thickets." The local names, brazilwood tree and brazil bloodwood tree, refer to the deep-red heartwood which Gentry notes is used to prepare a "light-red dye or ink" and also in fashioning rosary crosses. Martinez (2) adds the information that a decoction of the wood is used for "washing teeth." The native Indians call the plant "huchachago."

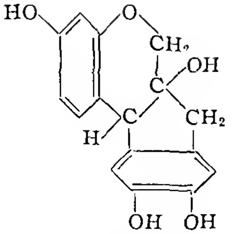
The local name, "palo de brazil," apparently has no geographic connotation concerning source, but rather is said to be derived from the word "braza," signifying "glowing like fire." Thus, the same common name is applied to a number of soluble-pigment-bearing red woods produced by different species of leguminous trees that grow in the East Indies, South and Central America, and Africa (3). Therefore, the plant is not to be confused with its relatives, *Caesalpinia echinata*, the brazilwood commonly found in the literature, or *C. brasilensis* and *C. crista*, the sources of brasiletto (4). However, all four plants are closely related botanically and chemically to each other and to *Haematoxylon campechianum*, the source of logwood and of the stain hematoxylin.

The red heartwood of *H. braziletto*, *C. echinata*, and *C. brasilensis* contains the water-soluble substance, brazilin (I) which upon mild alkaline oxidation or exposure to air yields the pigment red brazilein (II).

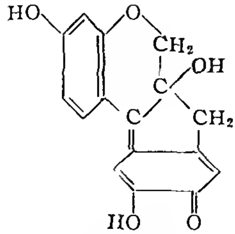
Search of the literature failed to reveal any reference to antibacterial action of constituents of *H. braziletto* and only inferential evidence concerning its relatives in the genera *Haematoxylon* and *Caesalpinia*. The 1899 edition of the U. S. D. (4) stated that logwood is "well adapted to the treatment of that relaxed condition of bowels which is apt to succeed *Cholera infantum*" and ascribed the effect to astringent action. The extract, which Hobbs' Botanical Handbook of 1876 (5) listed as an astringent tonic, was official as *Extractum Haematoxyli* in the National Formulary (6) until 1936. The isolated compound, hematoxylin has been reported to be "very feebly antiseptic" (7). The true brazilwood (*C. echinata*) and other similar woods, e. g., brasil-

* Received June 2, 1958, from the University of California, School of Pharmacy, San Francisco 22

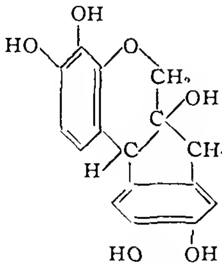
[1] From Loye H. Miller, Professor of Biology, Emeritus, University of California at Los Angeles



Brazilin (I)[2]



Brazilein (II)[2]



Hematoxylin (III)[2]

etto, sappanwood, and Nicaragua wood produced by other species of *Caesalpinia*, were once used medicinally but by the latter part of the nineteenth century were considered inert, and their medico pharmaceutical use was limited to coloring tinctures, etc. (4), and to preparation of mouthwashes and gargles (8).

EXPERIMENTS AND RESULTS

The first experiments were designed to simulate the native use of the red heartwood of *H. brazilelto*. Small chips (total weight 1.0, 0.6, 0.4, and 0.1 Gm., respectively) were placed in separate test tubes, each containing 10 ml. of tap water. The pH of the water was 4.0, and addition of the wood caused no change in pH, even after several days. The tubes were inoculated with a standard loopful of an active culture of *Salmonella typhosa* ATCC 6539. Duplicate tubes were similarly inoculated with *Escherichia coli*, ATCC 6522, and all tubes were incubated for eighteen hours at 37°. Each series was set up in duplicate. Parallel tubes were treated with chips of the sapwood. Results are shown in Table I.

TABLE I.—TURBIDITY OF TAP WATER CONTAINING WOOD OF *Haematoxylon brazilelto* AND INOCULATED WITH *S. typhosa* AND *E. coli* (INCUBATION 18 HOURS AT 37°)

Amt. of Wood Gm.	*S. typhosa* Heart wood	*S. typhosa* Sap wood	*E. coli* Heart wood	*E. coli* Sap wood
None (Control)	+++	+++	+++	+++
0.1	+++	+++	+++	+++
0.4	Clear	+++	+++	+++
0.6	Clear	+++	+++	+++
1.0	Clear	++	+	+++

[2] The structure of brazilin (I) has been established with certainty; structures shown for brazilein (II) and hydroxybrazilin hematoxylin (III) are considered the most probable (3).

No organisms developed when transfers were made from the clear tubes into nutrient broth and incubated at 37°, and it was concluded that the heartwood contains a water-soluble constituent which, in appropriate concentration, is bactericidal—not merely bacteriostatic—for *S. typhosa*. The results suggested that there might be a sound basis for the empirical native use of the heartwood and prompted more rigorous tests.

Ten grams of the granulated heartwood was packed carefully into a small percolator and 50 ml. distilled water was added. After twelve hours of maceration, the percolate was removed slowly, shell frozen in a suitable vessel, immersed in a mixture of dry ice and acetone, and then lyophilized. Complete drying usually required twelve to fifteen hours of lyophilization. The resultant dry residue consisted of orange-salmon pink, fluffy flakes that were soluble in distilled water to the extent of 33 mg./ml. Ten grams of heartwood yielded 120 to 130 mg. of dry residue from the first percolate.

Difco beef extract broth (14.9 ml.) containing different amounts of the dissolved extract was inoculated with 0.1 ml. of a culture of *S. typhosa*[3] and the cultures were incubated at 37° in inclined roller tubes rotating at a rate of 5 r. p. m. Periodically, the transmittance of the tubes (run in duplicate in each experiment) was determined by means of a Lumetron. In some experiments, hematoxylin[4] and brazilin[5] were studied comparatively with the wood percolate. Because of differences in color of broth containing different concentrations of extract or of the stains, it was necessary to run a separate uninoculated standard for each concentration of each product. The culture medium (pH 6.8) was well buffered, and even the highest concentration of extract or of the stains did not change the pH. Duplicate experiments were performed with *E. coli* and with *Micrococcus pyogenes* var. *aureus*.

Averaged results of several experiments are presented in Table II. Tubes with an optical density of 0.0 to 0.02 are visually clear. Thus, using visual criteria, one would consider the end point of both the crude extract and of hematoxylin to be between 0.25 and 0.5 mg./ml. for *S. typhosa* and between 0.125 and 0.25 mg./ml. for *M. pyogenes* var. *aureus*. Brazilin was slightly more antagonistic to the bacteria, the end point against *S. typhosa* and *M. aureus* being one tube higher in the dilution series. Failure of organisms to develop when transfers into drug-free broth were made from tubes with a reading of 0.01 or less suggests that the compounds were cidal, not merely static, in the stated concentrations.

E. coli was more sturdy than either of the other organisms and could neither be killed nor permanently inhibited even at the highest concentration tested (0.5 mg./ml.), although growth was suppressed for several hours. Cultures of this organism remained visually clear (o. d. = 0.02 or less) for three hours after inoculation into broth containing 0.0625 mg. of *H. brazilelto* extract or of brazilin per ml., and for six hours when inoculated into

[3] Eighteen-hour culture adjusted to a transmission of 47% as determined in Pyrex tubes (18 mm. i. d.) in a Lumetron Model 402 EF equipped with a neutral filter.
[4] National Aniline Division of Allied Chemical and Dye Corporation certification No. NH3.
[5] Histological stain from Hartman-Leddon Company C. I. No. 1243.

TABLE 11.—PER CENT TRANSMISSION AND OPTICAL DENSITY OF BROTH CULTURES OF THREE SPECIES OF BACTERIA INCUBATED FOR TWENTY-FOUR HOURS AT 37° IN THE PRESENCE OF *H. brasiletto* HEARTWOOD, EXTRACT OF HEMATOXYLIN, AND OF BRAZILIN

Treatment	*S. typhosa* T, %	O. D.	*E. coli* T, %	O. D.	*M. pyogenes* var. *aureus* T, %	O. D.
Control	5 0	1.30	4.6	1.34	2.0	1.70
Extract, mg./ml.						
0.5	98.1	0.01	Not tested		100.0	0.00
0.25	17.0	0 77	2.3	1.64	100 0	0.00
0.125	8.9	1 05	2 9	1 54	71 1	0 15
0.0625	2 5	1 60	2 9	1 54	31 9	0.50
Hematoxylin, mg./ml.						
0.5	96.7	0.01	2 6	1 59	98.6	0.01
0.25	19.5	0 71	2 5	1 60	99.0	0 00
0.125	14.7	0 83	5 5	1 26	17.2	0 76
0.0625	16 3	0.79	5.5	1.26	11.3	0 95
Brazilin, mg./ml.						
0.5	100	0.00	5.0	1.30	100	0 00
0.25	100	0.00	3.9	1.41	100	0.00
0.125	33.3	0.48	3 9	1 41	100	0 00
0.0625	17 8	0.75	4.2	1.38	97.4	0 01
0.03125	Not tested		Not tested		91.2	0 04
0.015625	Not tested		Not tested		10 6	0 97

broth containing the same concentration of hematoxylin. Control cultures usually showed some visible turbidity at the end of the first or second hour.

M. pyogenes var. *aureus* was the most easily affected of the organisms that were studied. Even after twenty-four hours of exposure to the lowest concentration of extract or of hematoxylin tested (0.0625 mg./ml.) growth in the experimental cultures was visibly considerably less than in the controls. The effect was particularly pronounced in cultures exposed to brazilin which, at that concentration, completely suppressed growth and almost completely suppressed it at 0.03125 mg./ml.

In agar-incorporation tests neither the extract (in concentrations from 0.25 to 4.0 mg./ml.) nor hematoxylin (in concentrations from 0.25 to 1.0 mg./ml.) evidenced antimycotic activity against *Penicillium notatum* or *Monilia fructigena*. However, the extract, at 2 mg./ml., prevented growth of *Trichophyton mentagrophytes* for four days and partially inhibited growth for ten days. At a concentration of 4 mg./ml., the extract completely suppressed growth of the organism for ten days, at which time the tests were terminated. Hematoxylin at levels from 0.25 to 1.0 mg./ml. partially inhibited growth of *T. mentagrophytes* for four days but not for longer test periods.

Successive extracts were made from each portion of granulated heartwood by adding 50 ml. distilled water to the percolator immediately after the previous percolate had been removed. Usually the yield from each of the first two percolates from a given aliquot of wood was between 120 and 130 mg. of dry, fluffy lyophilate. With succeeding percolations, the yield dropped until the 14th to 15th percolates yielded only 8 to 10 mg. of lyophilate. The total recovery in 15 extractions of 10 Gm. of heartwood was 600 to 650 mg. of lyophilate for a yield of 6 to 6.5%. There were no obvious physical differences in the appearance, either gross or microscopic, of the products obtained from the first and last extractions. But with succeeding extracts, beyond the second or third, there was a decrease in water solubility and in antibacterial activity. Lyophilates from the first two extractions of a given sample were active; those from fourth or later extractions were inactive.

Spectrophotometry failed to reveal significant differences between solutions of the active and inactive extracts, whether they were examined in acid, neutral, or alkaline media. Typical curves for solutions at pH 6.0 showed a primary absorption peak in the ultraviolet at 252 mμ and a secondary one at 285 mμ. A broad primary peak occurred in the visible range at 440 to 450 mμ and a secondary peak occurred at 520 mμ. There was no correlation between the order of the curves and the activity of the extracts. For example, in a typical experiment with a strongly bactericidal solution, a completely inactive solution, and one with intermediate activity, the absorbance in the ultraviolet range, in decreasing order, was active solution, inactive solution, and intermediately active solution. But in the visible wavelengths the decreasing order of absorbance was inactive solution, most active, and intermediately active. And in the infrared region, the curves of all three solutions were virtually identical, both qualitatively and quantitatively. Although the extinction coefficients at a given wavelength were different for the different solutions, the ratios of primary and secondary peaks in the ultraviolet and again in the visible were the same for the three solutions.

Solutions of brazilin exhibited only one peak in the ultraviolet, namely, at 285 mμ, and there was no absorbance in the visible. The absorbance of a 1.0 mg. % aqueous solution (pH 6.0) of brazilin at that wavelength was 0.223 with 1 cm. path length. The corresponding value for the active extracts was 0.220. There was little change upon acidification to pH 1.5 with HCl, the corresponding values at 285 mμ being 0.214 for brazilin and 0.216 for the extracts. In alkaline media (pH 10.0) a broad band with a distinct peak at 520 mμ was prominent. The absorbance at that wavelength was 0.948 for brazilin (1 mg. % at 1 cm. path length) and 0.191 for the active extracts. However, the compounds

were unstable in alkaline solution Upon restoration of the original pH, the pattern of absorption was quite different from that obtained originally and the curves produced upon subsequent realkalinization were entirely different from those just described

Paper chromatography was disappointing All of the extracts yielded two spots one which was easily visible and one which was only faintly visible in white light but both of which fluoresced brilliantly under ultraviolet irradiation These pairs of spots had identical R_f values in the bacteriologically active, moderately active, and inactive extracts Ultraviolet scanning failed to reveal any additional spots not detectable in white light Similar spots, of different intensity and fluorescence but with identical R_f values, were produced by solutions of hematoxylin The pairs of spots in all the preparations increased in intensity upon standing in air, and they were attributed to the pigments Brazilin produced a single spot which appeared to be qualitatively identical with one of the spots obtained with the extracts, but much more intense Tests with mixtures of brazilin and the extracts in varying proportions failed to separate the spots, suggesting that the extracts contained small amounts of brazilin

DISCUSSION

The failure of spectrophotometry to reveal qualitative or systematically graded quantitative differences among bacteriologically active, moderately active, and inactive solutions of the extracts suggests that the action of the effective solutions was not due directly to the pigments they contained and raises the possibility that the activity was due to some other compound or compounds However, although extensive chromatographic studies were not made, the trials which were performed failed to provide evidence of such a compound(s) and seemed to indicate qualitative chromatographic equivalence of the active, moderately active, and inactive extracts The similarity of chromatographic patterns developed by solutions of bacteriologically active and inactive extracts and of hematoxylin suggests again that the activity was not associated with the pigments

On the other hand, despite the above evidence, it is difficult to suppress the idea of a relationship between the pigment and the observed antibacterial action Tending to point to such a connection are the facts that (*a*) extracts prepared from the unpigmented sapwood and bark were completely inactive against the test organisms, (*b*) the aqueous extracts prepared from the heartwood would be expected to contain brazilin, (*c*) despite some differences, aqueous solutions of brazilin and of the extracts had spectrophotometric similarities, (*d*) brazilin and hematoxylin have closely related phenolic structures (the latter being simply hydroxybrazilin), (*e*) many phenols are known to be antibacterial, and (*f*) there was approximate concordance between the end point concentrations of the most active solutions of the *H brazilletto* extracts and of hematoxylin and of brazilin for inhibition of the test organisms The latter point could, of course, be simply a matter of coincidence But, in view of the close structural similarity of brazilin and hematoxylin, and of their products, brazilein and hematein, this does not seem plausible

Brazilin and hematoxylin and their oxidation products, brazilein and hematein, respectively, are extremely sensitive to exposure to light and air and to slight alterations in pH, particularly in alkaline or near alkaline media, and to other environmental conditions It seems possible, therefore, that they may exist in several isomeric forms which might not be distinguishable by spectrophotometric or chromatographic methods but which might, nevertheless, differ in their biologic properties Therefore, it is suggested that the observed antibacterial activity was, in fact, due to specific isomeric form(s) of the pigment The decrease in antibacterial activity of successive extracts prepared from a given sample of wood and the variation seen in a particular extract, e g, first, second, etc, from different samples of wood would, according to this working hypothesis, be ascribed to the existence of different proportions of bacteriologically active and inactive isomers in the different preparations

SUMMARY

1 In some areas of Mexico chips of the wood of the native plant, *Haematoxylon brazilletto*, are added to the drinking water and the infusion is used empirically as a tonic (and anti infective?) in some enteric disorders

2 The heartwood of this plant contains a water soluble compound which, in appropriate concentrations, is bactericidal for *Salmonella typhosa* and *Micrococcus pyogenes* var *aureus* and temporarily inhibits the growth of *Escherichia coli*

3 Solutions of the stains, brazilin and hematoxylin, derived from related plants, have a similar effect

4 It is suggested that the activity of the extracts is due to one or more isomeric forms of brazilin and/or its oxidation product, brazilein

5 Extracts prepared from the sapwood and from the bark of *H brazilletto* were inactive antibacterially

6 None of the preparations exhibited antifungal activity against *Monilia fructigena* or *Penicillium notatum*, although both hematoxylin and *H brazilletto* temporarily retarded growth of *Trichophyton mentagrophytes*

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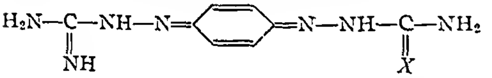
A Note on Condensation Reactions of N-Guanidino-*p*-benzoquinone Imine Hydrazine Derivatives*

The *in Vivo* Antibacterial Activity of One Derivative

By RICHARD E. STRUBE and CHARLES LEWIS

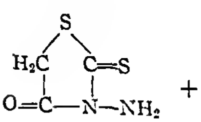
The condensation product of 3-hydrazino-4-amino-5-mercapto-1,2,4-triazole with N-guanidino-*p*-benzoquinone imine was active in mice infected with *S. hemolyticus*. 3-Aminorhodanine, methyl dithiocarbazinate, 2-methylthiosemicarbazide and a number of 4-amino-1,2,4-triazole derivatives did not condense with N-guanidino-*p*-benzoquinone imine.

A PREVIOUS PUBLICATION from this laboratory described the condensation of 3-aminorhodanine (I) with aromatic and heterocyclic aldehydes (1). It was shown that in an acid medium reaction occurred with the amino group and not with methylene group of 3-aminorhodanine. Domagk's discovery of the activity of certain quinone derivatives in mice against Streptococci, in particular the compounds formulated below (2, 3), prompted the study of the reaction of 3-aminorhodanine with N-guanidino-*p*-benzoquinone imine (II) in the

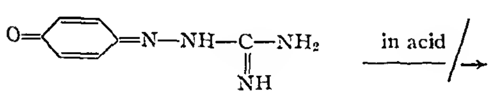


X = S, O, or NH

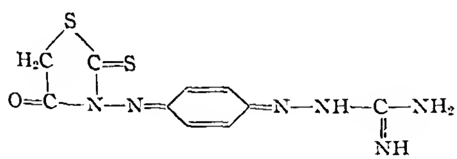
expectation of obtaining compound III. However, all attempts to make this compound were



I

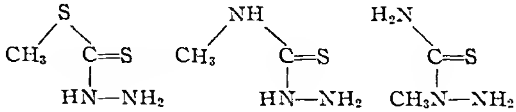


II



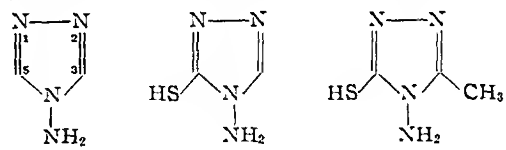
III

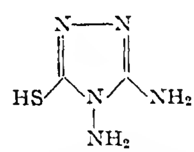
unsuccessful. The condensation of II with methyl dithiocarbazinate (IV), an open-chain analog of I, also failed. The desired condensation occurred readily with 4-methylthiosemicarbazide (V), a nitrogen analog of IV. These few experimental results suggested that failure of condensation of 3-aminorhodanine was due to the effect of the ring sulfur atom. However, additional experi-



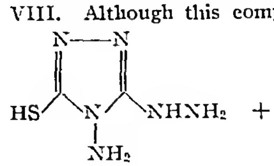
IV V VI

ments showed that this was not the only factor involved. In general, the disubstituted hydrazino group $>N\text{-}NH_2$, which is also present in 3-aminorhodanine, is unable to condense with II. This assumption was supported by the study of the condensation reaction of a few other disubstituted hydrazine derivatives. As already mentioned, condensation occurred with the monosubstituted hydrazine (V) but the disubstituted analog (VI) (4) did not condense. A number of 4-amino-1,2,4-triazoles, such as 4-amino-1,2,4-triazole (5), 4-amino-5-mercapto-1,2,4-triazole (6), 3-methyl-4-amino-5-mercapto-1,2,4-triazole (7), and 3,4-diamino-5-mercapto-1,2,4-triazole (8), did not condense with II.

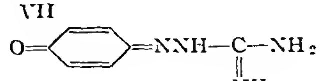


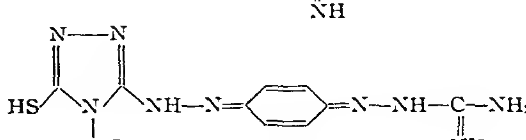


A condensable triazole was obtained by introducing a monosubstituted hydrazine group. This compound, 3-hydrazino-4-amino-5-mercapto-1,2,4-triazole (VII), could be prepared in higher yields than reported in the literature (5, 9) by allowing diethylxanthate to react with an excess of hydrazine. Condensation of this thiazole with II gave compound VIII. Although this compound had a low order



VII





VIII

* Received May 9, 1958, from the Research Division, The Upjohn Company, Kalamazoo, Mich.
The authors wish to thank Dr. J. B. Wright and Mr. D. A. Lyttle of the Department of Chemistry of The Upjohn Company for the preparation of a few thiazole derivatives.

TABLE I.—COMPARISON OF *in Vivo* ANTIBACTERIAL ACTIVITIES[a] OF COMPOUND VIII WITH N-GUANIDINO-N'-THIOUREIDO-*p*-BENZOQUINONE DIIMINE NITRATE IN INFECTED MICE

Organism	Compound VIII, Mononitrate Oral	i. p.	s. c.	Domagk's Compound Oral	i. p.
S. hemolyticus C203	87	*ca.* 80	*ca.* 320	<40	<25
D. pneumoniae I Felton	>800	...	>320	>80	...
K. pneumoniae A-D	>800	>80	...	>80	>25
M. aureus 284	>800	...	...	...	...
P. multocida 449	>800	..	>320	50–100	...
Tolerated dose	>800	80–100	>320	50–100	25

[a] Expressed as median protective doses in mg /Kg.

of activity against a series of bacteria in the agar-dilution plate test method, it was active in mice against *S. hemolyticus* when administered by the oral, subcutaneous, or intraperitoneal route.

EXPERIMENTAL

3 - Hydrazino - 4 - amino - 5 - mercapto - 1,2,4-triazole.—Thirty cubic centimeters of hydiazine (95%), 37.5 cc. of hydrazine hydrate, and 7.5 cc. of water were placed in a 250-cc., 3-neck, round-bottom flask provided with a stirrer, a reflux condenser, a dropping funnel, and a gas inlet tube for nitrogen. The system was swept out with a stream of nitrogen which was allowed to run until the product was isolated. The flask was placed in a water bath maintained at 60° and 75 Gm. (0.50 mole) of diethylxanthate was added to the well-stirred solution over a period of thirty minutes. Ethyl mercaptan, evolved rapidly, was absorbed by traps, one containing an excess of 30% sodium hydroxide, and the following one containing mercuric chloride and cadmium carbonate. The reaction mixture was stirred for an additional thirty minutes at 60°. During this period the condenser was maintained at 30° to allow ethyl mercaptan to escape. The flask was heated for one and one-half hours on a steam bath, with stirring. After cooling to room temperature, 250 cc. of water was added. The pH was adjusted to 6 with concentrated hydrochloric acid (35 cc. required) and the total volume was adjusted to 400 cc. with water. The mixture was heated to boiling and filtered while hot. The solid was washed with boiling water made slightly acid with hydrochloric acid. The solid was dried *in vacuuo* at 60°. The yield was 25.1 Gm., m. p. *ca.* 221°, decompn. This material was purified by treating with 200 cc. of *N* NaOH plus 60 cc. of water. The insoluble material was removed by filtration and the clear filtrate was acidified by the addition of 18 cc. of glacial acetic acid. The solid formed was collected and washed with water and alcohol. After drying *in vacuo* at 60°, the white product weighed 24.3 Gm. (66.6% yield), m. p. 222–228°, decompn.

Condensation Product VIII.—N-Guanidino-*p*-benzoquinone imine (8.20 Gm., 0.05 mole) was dissolved in a solution of 65 cc. of water and 14.5 cc. of concentrated hydrochloric acid at room temperature. To this solution was added a solution of 3-hydrazino-4-amino-5-mercapto-1,2,4-triazole (7.26 Gm., 0.0497 mole) in 118 cc. of water and 25.4 cc. of concentrated hydrochloric acid at room temperature. The mixture was filtered after forty-eight hours and the dark red solid was washed, in two portions, with 100 cc. of water made acid with 2 cc. of concentrated hydrochloric acid. The product was purified by recrystallization from a minimum of boiling, slightly acid water (approx. 1,400 cc. required, prepared by adding 20 cc. of concentrated hydrochloric acid to 1,600 cc. of water). After drying *in vacuo* over sodium hydroxide at room temperature, 9.8 Gm (60%) of compound VIII was obtained as the hydrochloride.

Anal. Calcd. for $C_9H_{14}ClN_{10}S$: C, 32.88; H, 3.98; Cl, 10.78; S, 9.75. Found: C, 32.99; H, 3.81; Cl, 10.67; S, 9.80.

The mononitrate of compound VIII was prepared in a somewhat similar way using nitric acid instead of hydrochloric acid.

Antibacterial Activity in Mice.—The procedure for testing compounds in infected mice has been described previously (10). Male CF-I mice were infected with 100 LD_{50}'s of the organism to be studied and the infected animals were treated on three successive days with the compound. At the end of seven days, the efficacy of the compound was judged by the mortality ratio of the treated animals as compared to the mortality ratio of an untreated group.

Since compound VIII (nitrate) is rather insoluble in water, it was suspended in 0.25% Methocel.[1] The desired drug level was administered either subcutaneously or intraperitoneally in 0.2 cc. volume, or orally by intubation in 0.5 cc. volume.

Preliminary tests demonstrated that noninfected mice tolerated daily subcutaneous and oral doses of 320 mg./Kg. and 800 mg./Kg., respectively, without showing gross signs of toxicity. Pertinent results observed during the study of this compound are summarized in Table I. The original compound of Domagk (2) was also studied and the data are included for comparative purposes.

The data in Table I show that compound VIII was not toxic and that it protected *S. hemolyticus* infected mice when given by either the oral, subcutaneous, or intraperitoneal route. Its activity can best be demonstrated when given via the oral route. The activity of Domagk's compound as reported by him has been confirmed.

SUMMARY

1. 3 - Hydrazino - 4 - amino - 5 - mercapto - 1,-2,4-triazole condensed with N-guanidino-*p*-benzoquinone imine.

2. The condensation product is active in mice against *S. hemolyticus* when administered by the oral, subcutaneous, or intraperitoneal route.

[1] Dow Chemical Company, Midland, Mich.

3. 3-Aminorhodanine, methyl dithiocarbazinate, 2 methylthiosemicarbazide, 4-amino-1,2,4-triazole, 4 - amino - 5 - mercapto - 1,2,4 - triazole, 3 - methyl-4-amino-5-mercapto-1,2,4-triazole, and 3,4-diamino-5-mercapto-1,2,4-triazole did not condense with N-guanidino-*p*-benzoquinone imine.

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Book Notices

Analytical Abstracts. A monthly publication of The Society for Analytical Chemistry, 14 Belgrave Square, London, S. W. 1., England.

Analytical Abstracts, first published in January 1954 on the cessation of publication of *British Abstracts C, Analysis and Apparatus*, covers the analytical literature of the world.

It contains upwards of 4,000 abstracts per annum prepared by abstractors who are experts in particular fields of analytical chemistry. Many abstracts are prepared by chemists in the countries of publication of the original papers. Analysts will appreciate the advantage of having all abstracts dealing with their subject in a single publication.

Analytical Abstracts can be obtained from January 1, 1959 for $14 per annum postage-free including index. Some earlier volumes are still available. The subscription rate including *The Analyst* is $23.52 per annum postage-free, including both indexes. *Analytical Abstracts* is also available printed on oneside of the paper, so that abstracts can easily be incorporated into existing reference systems. Price per annum without index $15.05; with index $18.48.

How to Live With Diabetes. By HENRY DOLGER, M.D. and BERNARD SEEMAN. W.W. Norton & Co., Inc., New York, 1958. 192 pp. 14 x 21 cm. Price $3.50.

A book designed for diabetics and those concerned with the disease. The major emphasis is on the person with diabetes, and covers much information which he should have available. The book can conscientiously be recommended by pharmacists to diabetics.

Experimental Pharmacodynamics. 1958 rev. By T. KOPPANYI and A. G. KARCZMAR. Burgess Publishing Company, Minneapolis, 1958. xvi + 258 pp. 21 x 27.5 cm. Price $5.50 Paperbound. Printed by photo offset.

This brief outline attempts to acquaint the student with the experimental approach to pharmacology and pharmacodynamics. It is designed to serve both as a laboratory outline and textbook on pharmacology.

Nederlandse Pharmacopee. Zesde Uitgave. Ministerie Van Sociale Zaken En Volksgezondheid, 1958. xxvii + 633 pp. 15.5 x 24 cm.

This new edition of the Nederlandse Pharmacopee represents a complete revision. The drugs for which specifications are provided are for the most part older therapeutic agents and pharmaceutical vehicles and necessities. Apparently no drugs restricted by patents have been included in this edition. This is marked contrast to the latest lists of admissions to the United States Pharmacopeia and the National Formulary.

Biochemical Preparations. vol. 6. Edited by Carl S. Vestling. John Wiley & Sons, Inc. New York, 1958. ix + 105 pp. 14.5 x 23 cm. Price $5.25.

Carefully checked procedures are presented for the preparation of crystalline animal cytochrome *c*, deoxyribonucleic acid, 2,3-diphosphoglyceric acid, *l*-α-glycerophosphorylcholine, 3-hydroxyanthranilic acid, β-hydroxy-β-methylglutaric acid, insulin, lanosterol from "isocholesterol," leucine aminopeptidase, α-methylserine and bis(hydroxymethyl) glycine, crystalline horse oxyhemoglobin, old yellow enzyme, crystalline papain and benzoyl-*l*-argininamide, crystalline muscle phosphorylase *a* and *b*, phosphoserine, ribonucleic acid from rat liver, ribulose diphosphate, and *dl*-tryptophan-7a-C^{14}.

Practical Clinical Biochemistry. 2nd ed. By HAROLD VARLEY. Interscience Publishers Inc., New York, 1958. viii + 635 pp. 14 x 21.5 cm. Price $6.50.

This book describes procedures of the many biochemical tests carried out in hospital laboratories as an aid to the diagnosis and treatment of disease. It is designed to be used primarily as a practical textbook.

Medizinal Kalender 1959. W. v. Brunn. Georg Thieme Verlag, Stuttgart, Germany, 1958. xx + 856 pp. 9 x 16 cm. Price DM 9.60. Distributed in the U. S. and Canada by Intercontinental Medical Book Corp., New York. Price $2.30.

A handbook of diagnoses and therapy of especial interest to physicians.

Kurzes Lehrbuch Der Pharmazeutischen Chemie, Funfte Auflage By K. BODENDORF Springer Verlag, Berlin W 35, Reichpietschufer 20, West Berlin, Germany, 1958 vii + 490 pp 16 5 x 25 cm Price DM 34 50

Textbook of inorganic and organic chemistry designed for pharmacy and medical students

Russian English Medical Dictionary By STANLEY JABLONSKI Academic Press, Inc, New York, 1958 x + 423 pp 15 x 23 cm Price $11

This dictionary is a feature of the Russian Scientific Translation Program conducted by the National Institutes of Health, U S Public Health Service It is designed to assist in the translation of medical and allied books and papers written in the Russian language

Simple Methods of Contraception Edited by Winfield Best and Frederick S Jaffe Planned Parenthood Federation of America, New York, 1958 64 pp 15 5 x 23 5 cm

Various simple contraceptive techniques are described and evaluated and their medical, social, and moral implications are discussed

Chemical Transformations by Microorganisms By FRANK H STODOLA John Wiley & Sons, Inc, New York, 1958 ix + 134 pp 12 5 × 18 5 cm Price $4 25

This book is based on the second series of E R Squibb Lectures on Chemistry of Microbial Products, Rutgers University The production of new and useful chemicals by microorganisms is discussed A full account of the production of alpha ketoglutaric acid and L glutamic acid by fermentation is presented How microorganisms are used in industry and recent information on research on mycobacterium tuberculosis are also included in the lectures

Tuberkulose Bucherei Monographien zur Monatsschrift Der Tuberkulosearzt Georg Thieme Verlag, Stuttgart, Germany, 1958 152 pp 16 5 x 24 cm Price DM 17 80 Distributed in the United States by Intercontinental Medical Book Corporation, New York Price $4 25

A series of papers presented on the occasion of the dedication of the Robert Koch Tuberculosis Clinic of the University of Freiburg, in February 1958 The lectures cover the general field of tuberculostatic agents and the development of resistant strains of the tubercle bacillus

Serological and Biochemical Comparisons of Proteins Edited by William H Cole Rutgers University Press, New Brunswick, 1958 xi + 119 pp 15 5 x 23 5 cm Price $2

Seven papers are reproduced in this paper bound book which were presented during a program of studies on protein metabolism sponsored by the Bureau of Biological Research, Rutgers University, New Brunswick, N J The papers are designed to cover recent developments in the basic mechanisms that are concerned with protein interactions in the living organism

The Guinea Pig in Research By MARY ELIZABETH REID Human Factors Research Bureau, Inc, Washington 4, D C, 1958 87 pp 15 x 23 cm Price $2

This paper-bound booklet describes the known dietary needs of the guinea pig in quantitative terms together with a brief account of some aspects of its physiology which may be of interest to the nutritionist and may increase its usefulness in medical research

A Contribution to the History of Ancient Transportation and Trade By W H BLOME Ann Arbor Publishers, Ann Arbor, 1958 vi + 86 pp 21 5 x 28 cm Paperbound Printed by photo offset

The author of this book presents data pertaining to ancient transportation, land routes of commerce, travel, transport by water, merchandise transported, with special emphasis on drugs, and the dangers and difficulties that were encountered The author was formerly Chief Pharmacist with Frederick Stearns and Company and later Professor of Pharmacy at Wayne State University of Detroit He is now living in retirement in Manchester, Mich

Experimental Pharmacognosy 2nd ed By VARRO E TYLER, Jr, and ARTHUR E SCHWARTING Burgess Publishing Co, Minneapolis, 1958 iii + 81 pp 21 x 27 cm Price $2 75 Paperbound Printed by photo offset

A laboratory outline designed to aid in teaching fundamental principles which are universally applicable Biochemical processes are stressed, and a key for the identification of powdered drugs is included

Vade Mecum Du Veterinaire By A BRION Vigot Freres, Editeurs, Paris, 1958 xx + 752 pp 10 5 x 17 5 cm Price 4,200 fr

A presentation in capsule form of present day knowledge in most of the fields which interest the practicing veterinarian

XVIIe Congres Des Sciences Pharmaceutiques Leiden, 1957 Conferences et Communications D B Centen, Amsterdam, 1958 351 pp 16 x 24 cm Price 195 fr

A series of papers and discussions presented at the Seventeenth Congress of the Pharmaceutical Sciences held in Leiden in 1957 Most of the papers are written in French, German or English, and the authors represent several nationalities

Abrege de Matiere Medicale Matieres premieres d origine vegetale 3rd ed By M R PARIS and H MOYSE Vigot Freres, Editeurs Paris, 1958 196 pp 16 x 24 cm Price 1,100 fr

This book (in French) presents information in abstract form on material of vegetable origin used in materia medica A classification of vegetable drugs according to their pharmacodynamic action is included and a subject index is appended

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII | *FEBRUARY 1959* | NUMBER 2

A Kinetic Study of the Specific Hydrogen Ion Catalyzed Solvolysis of Chloramphenicol in Water-Propylene Glycol Systems*

By ARNOLD D. MARCUS† and ANTHONY J. TARASZKA

The specific hydrogen ion catalyzed degradation of chloramphenicol in solutions containing up to 50 per cent (v/v) propylene glycol is qualitatively similar to degradation in purely aqueous systems. The reaction remains pseudo-first order with respect to chloramphenicol and the direct dependence of the rate upon acid concentration is maintained. The contributions of a water reaction at low acid concentrations is still evident in 10 per cent glycol systems but not in 30 or 50 per cent glycol. If the previously postulated mechanism for this reaction is correct, the present data are in conflict with the requirements of the Bronsted-Christiansen-Scatchard equation for a reaction between ions of like sign. In the present study a decrease in dielectric constant resulted in an increase rather than a decrease in the rate of the reaction. The inverse relationship between rate and dielectric constant is rationalized by assuming the reaction to be of the ion-dipole rather than of the ion-ion type. This assumption appears to receive support from the qualitative agreement between the data and the requirements of the Amis equation and a mechanism is offered to account for the postulated ion-dipole reaction. The activation energies for the reactions in 10, 30, and 50 per cent propylene glycol are 21.0, 20.5, and 20.0 kilocalorie/mole, respectively. The direct relationship between activation energy and dielectric constant agrees with the theoretical requirements for a cation-dipole reaction.

THIS INVESTIGATION was undertaken in an attempt to study the specific solvent or other effects of propylene glycol upon the rate of hydrogen ion catalyzed degradation of chloramphenicol in water-propylene glycol solutions A major objective of this study was the critical and, if possible, quantitative evaluation of the effect of increasing glycol concentrations upon the rate of hydrolysis or other solvolytic cleavage, if any. Such a study appeared to be of particular importance in view of the widespread use of mixed solvents in pharmaceuticals and the relative lack of information concerning the effects of such solvent systems upon the stability of active ingredients

The hydrolytic, or other solvolytic, decomposition of pharmaceuticals is by no means a new problem. Drugs of such diverse chemical constitution as procaine, aluminum acetate, naphazoline, and chloramphenicol are all subject to hydrolytic breakdown (1–4) and these liabilities are often important in formulation procedures. In many instances solvolysis may be inhibited, or avoided entirely, by a variety of remedies including judicious maintenance of pH (1, 2), "tying up" the labile species in the form of a stable complex (5), the use of an insoluble form of the active ingredient (6), or by careful control of buffer capacity and constituents (7).

Despite the apparent universality of the above procedures, it is often found that their application fails to achieve the desired resistance to solvolysis.

* Received May 3, 1957, from College of Pharmacy, Rutgers University, Newark 4, N J

Presented to the Scientific Section, A PH A, New York Meeting, May, 1957

This study was supported, in part, with funds supplied by the Research Council of Rutgers University

Portions of this paper are adapted from the prize-winning manuscript submitted by Anthony J Taraszka in the Eastern Region, 1956, Lunsford Richardson Pharmacy Award competition

† Present address Merck Sharp and Dohme Research Laboratories, West Point, Pa

We often find, therefore, that the problem is attacked by a partial or complete change in the solvent system It has been recently reported (8), for example, that the stability of pentobarbital in solution is materially enhanced by the use of a polyethylene glycol solvent system Similar reasoning has been successfully applied in the official monographs on tribromethanol solution, isofluophate solution, nitrofurazone solution (9–11), and other preparations

It is probably as a result of these and similar successes that there is a generally held opinion that the use of nonaqueous solvents to replace all or a portion of, the water in a solution is some sort of panacea Such an assumption is largely erroneous and probably results from a lack of appreciation of the ability of nonaqueous solvents, especially those which are hydroxylic, to participate in, or otherwise influence, solvolytic reactions Many of the claims which have been advanced in this area are either without sound foundation in experimental fact or are the results of purely superficial evidence Water may have been branded a villain too often without good cause

It is probable that this situation has resulted, at least in part, from the scarcity of reliable chemical kinetic studies regarding the rates of degradation of pharmaceuticals An accurate evaluation of the specific or other contributions of a second solvent is difficult, if accurate and precise studies of degradation in water have not been carried out

As part of a project concerning the effects of nonaqueous solvents upon the stability of pharmaceuticals, it was decided to study, initially, propylene glycol This solvent is finding increasing use and is of such a chemical constitution that it might very possibly participate in solvolytic reactions Since only a few drugs have been evaluated with respect to their behavior in aqueous systems, it was decided that it would be most prudent to choose for this study, a drug which decomposed solvolytically in acid systems, thus eliminating the need for buffer systems and pH measurements, both of which may have little significance in mixed solvents Inasmuch as the response of chloramphenicol, in purely aqueous systems, to temperature and pH has been almost completely evaluated, this antibiotic was chosen for investigation

Since it was possible that the inclusion of propylene glycol in the reaction media might alter the order and/or character of the degradative reactions or affect the formation or interaction of ionic species, the reactions were carried out in water-propylene glycol systems containing up to 50 per cent (v/v) glycol The data obtained were analyzed with a view toward determining, if possible, the degrees of both hydrolytic and non-hydrolytic solvolysis (Figs 1 and 2) as well as the influence of any electrostatic effects

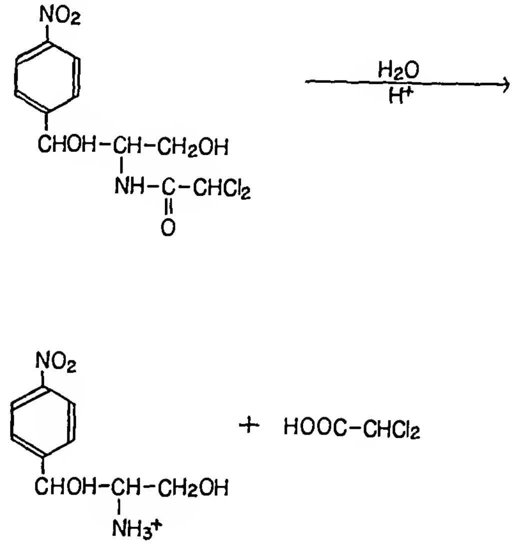


Figure 1

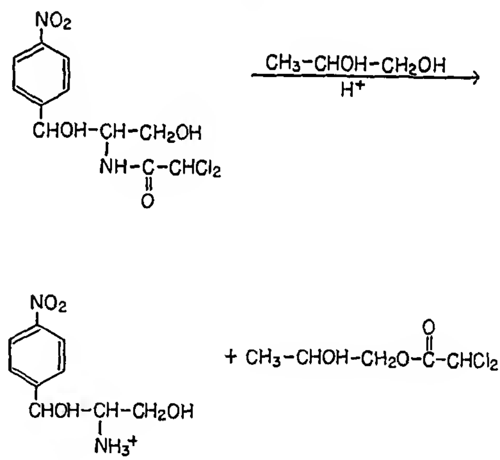


Figure 2

EXPERIMENTAL

Reagents —Other than the chloramphenicol and U S P grade propylene glycol, all chemicals were reagent grade

Method of Degradation —The general experimental procedure of Higuchi, *et al* (7, 12), was employed to effect all degradations The glycol perchloric acid solutions were prepared by adding the glycol to the standard solution of perchloric acid, dropwise, through a 25 ml buret This procedure minimized any errors which might have resulted from the viscosity of the glycol

Analytical Procedure —Partition chromatographic elution of residual undegraded chloramphenicol followed by spectrophotometric determination of the antibiotic as reported by Higuchi, *et al* (13),

was employed throughout, with the following modifications: The mobile phase was changed from 10 to 15% (v/v) ethyl acetate in chloroform and all spectrophotometric measurements were made at 272 mμ, the peak for chloramphenicol in this solvent. In addition, the samples of reaction mixture containing 50% glycol were diluted with an equal volume of purified water prior to separation.

DISCUSSION OF THE EXPERIMENTAL RESULTS

Nature of the Acid Catalyzed Reaction.—It is evident from Fig. 3 that the addition of propylene glycol to the reaction systems in no way affects the pseudo-first order character of the overall degradative reaction. While this evidence is not conclusive, the nature of these plots, along with the previously demonstrated behavior of chloramphenicol in purely aqueous systems, overwhelmingly indicates that here, too, solvolysis is the major degradative pathway. For this reason it will be implicitly assumed in the balance of the discussion that solvolysis represents the only significant route of degradation. It should be noted, however, that these plots cannot, except indirectly, indicate the degree to which propylene glycol influences the rate of degradation. The data, plots, and discussion to follow may serve to illuminate this area.

Previous studies (7, 12) concerning the degradation of chloramphenicol have shown that in aqueous systems below pH 7, three major types of reactions are responsible for degradation; general acid-base catalyzed hydrolysis, specific hydrogen ion catalyzed hydrolysis, and an uncatalyzed or "water" reaction. In highly acid systems, however, the rate of degradation depends only upon the temperature and acid concentration. In solutions where the concentration of acid is low (*ca.* 0.025 *M*), however, the total rate represents a summation of the hydrogen ion catalyzed reaction and the "water" reaction. As a result, the rate of hydrolysis of chloramphenicol is significant even at "zero" hydrogen ion concentration.

This behavior, plus the experimentally established increase in the solubility of chloramphenicol with increasing hydrogen ion concentration, led Higuchi and Marcus (12) to postulate that the hydrogen ion and the uncatalyzed solvolyses are associated with different substrates. These authors said that the substrate in the acid catalyzed reaction is a monoprotonated chloramphenicol with a positive charge on the substrate, whereas the substrate in the "water" reaction is chloramphenicol itself.

Figures 4, 5, and 6 show that the linear relationship between rate of degradation and acid concentration (above 0.025 *M*) is unaffected by including 10, 30, or 50% propylene glycol in the reaction mixtures. There is, however, a notable difference between the plots in Fig. 4 and those in Figs. 5 and 6, even at low concentrations of acid. If the lines in Fig. 4 were drawn so as to pass through all of the experimentally determined points, they would not pass through the origin (as drawn) but would curve or level off to intersect the ordinate significantly above the origin. In light of the preceding discussion concerning the

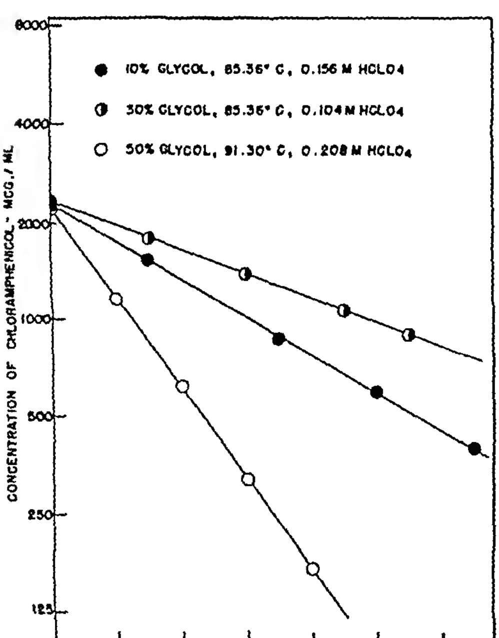


Fig. 3.—Plots showing the rate of hydrogen ion catalyzed hydrolysis of chloramphenicol to remain first order with respect to the antibiotic under a variety of experimental conditions

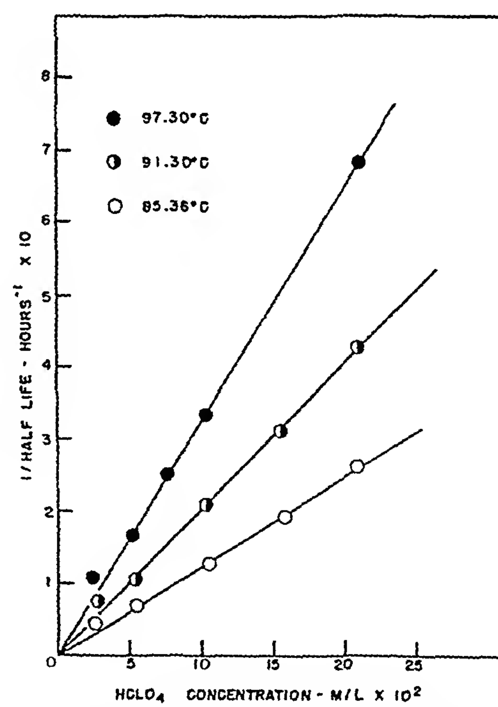


Fig. 4.—Plots showing the direct relationship between the rate of acid catalyzed degradation of chloramphenicol and acid concentration in 10% (v/v) propylene glycol. As noted in the text, the departures from linearity at low concentrations of acid may be ascribed to the contributions of the "water" reaction.

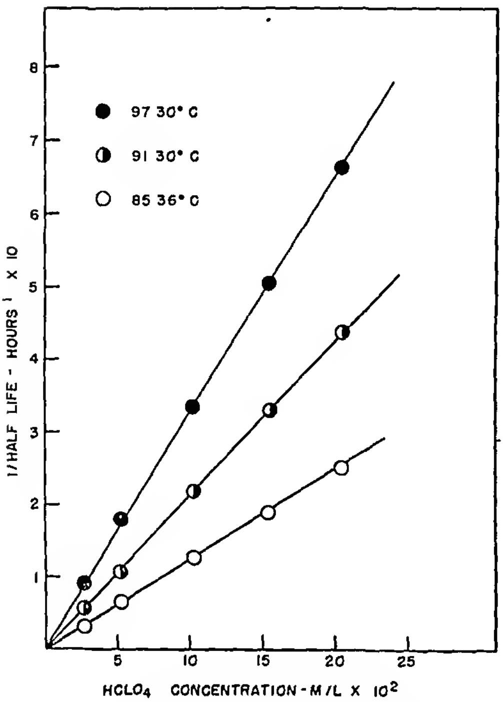


Fig 5—Plots similar to those in Fig 4 but for 30% (v/v) propylene glycol The lack of any noticeable deviations from linearity at low concentrations of acid indicate that the "water" reaction is either absent entirely or that its contributions are negligible

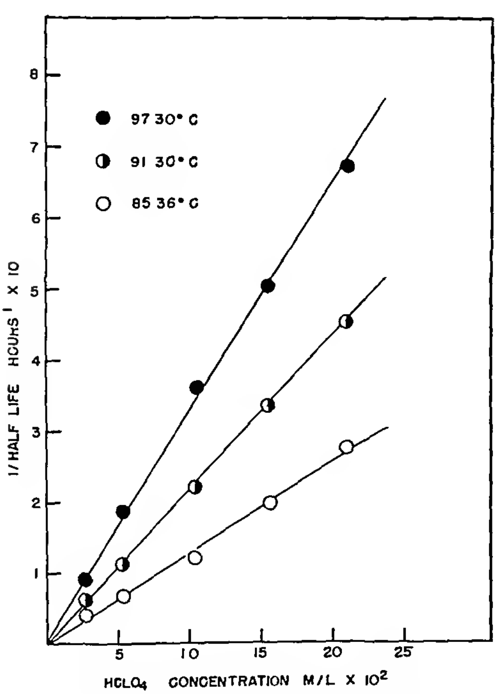


Fig 6—Plots similar to those in Fig 5 but for 50% (v/v) propylene glycol

dual degradative pathways, it is clear that in the solutions containing only 10% propylene glycol, the "water" reaction still contributes significantly at low hydrogen ion concentrations

In Figs 5 and 6, however, a much different picture is presented In these plots all of the experimentally determined points, including those obtained at 0 026 *M* acid, fall directly on the lines drawn to pass through the origin There is no discernible evidence of any levelling off It is therefore, possible to conclude that in reaction systems containing 30 or 50% propylene glycol and 0 026 *M* acid, the uncatalyzed reaction is either absent entirely or else plays so small a role as to be beyond the power of resolution of the analytical procedures employed One thing appears certain, in reaction systems containing higher concentrations of the glycol, the "water" reaction is substantially suppressed either as a result of simple decrease in the relative concentration of water, or because the glycol in some manner favors the mono protonated form of the substrate Mono-protonated chloramphenicol is, of course, virtually unaffected by the "water" reaction

Although the preceding discussion and supporting figures indicate that at low hydrogen ion concentrations the addition of propylene glycol tends to reduce the overall rate of degradation, a quite different view is gained upon inspection of the plots in Figs 4, 5, and 6 at higher acid concentrations While the qualitative character of the specific hydrogen ion catalyzed solvolysis of chloramphenicol is essentially unaffected by the inclusion of glycol in the reaction systems, a change in qualitative character was not actually anticipated It was expected, however, that the quantitative picture would be quite different In line with generally held opinions, we assumed that the rate of degradation would decrease significantly with increasing concentrations of propylene glycol This assumption was supported, from a theoretical standpoint, by the requirements of the Bronsted Christiansen Scatchard equation (14) for reactions between ions of like sign

We were, therefore, surprised to find that the inclusion of propylene glycol did not result in a decrease in the rate of the hydrogen ion catalyzed reaction Instead, it became apparent that an increase in glycol concentration caused an increase in the catalytic activity of the hydrogen ion and, therefore, in the rate of the reaction These small but definite increases are summarized in Tables I and II It should also be noted that there is an apparent lack of any regular relationship between the rate of reaction and the concentration of propylene glycol

DISCUSSION OF THE REACTION MECHANISM

It is possible to rationalize the increases in the rates of reaction with increasing concentrations of propylene glycol in several ways It seemed possible that upon adding glycol to the reaction systems there would be a competition between water and the glycol, so that the systems would contain both hydronium ions and glycolated protons If attack by a glycolated proton is facilitated by the newly increased possibilities for van der Waals' type interactions with the substrate, the increase in rate of degradation need not remain puzzling This proposal

TABLE I.—CATALYTIC CONSTANTS FOR HYDROGEN ION CATALYZED REACTION IN THE VARIOUS SYSTEMS

Temp., °C.	Glycol, %	k_H L. moles^{-1} hours^{-1}
97.30	0	2.00[a]
97.30	10	2.18
97.30	30	2.21
97.30	50	2.22
91.30	0	1.223[a]
91.30	10	1.433
91.30	30	1.469
91.30	50	1.490
85.36	10	0.845
85.36	30	0.875
85.36	50	0.902

[a] This value obtained from the data of Higuchi and Marcus (12).

TABLE II.—THE EFFECT OF THE CONCENTRATION OF PROPYLENE GLYCOL UPON THE RATE OF SOLVOLYSIS OF CHLORAMPHENICOL

Temp., °C.	Glycol, %	Half Life-Hours[a]
97.30	0	3.47[b]
97.30	10	3.17
97.30	30	3.14
97.30	50	3.13
91.30	0	5.67[b]
91.30	10	4.84
91.30	30	4.72
91.30	50	4.65
85.36	0	8.54[b]
85.36	10	8.23
85.36	30	7.93
85.36	50	7.69

[a] Half lives in 0.1 *M* perchloric acid.
[b] This value obtained from the data of Higuchi and Marcus (12).

is seriously set back, however, by the fact that at higher concentrations of the glycol (30 and 50%) the increase in rate does not begin to compare with the increase in glycol concentration. Since the existence of any substantial concentrations of glycolated protons would demand the presence of substantial amounts of propylene glycol, the fact that the rate of degradation is almost independent of glycol concentration in the range 10 to 50% glycol led us to abandon pursuit of this possibility.

In the course of our attempts to explain the apparent departure from expected behavior, the possibility was advanced that a glycolated proton would be a stronger acid than the hydronium ion, thus achieving a greater catalytic effect. The same deficiencies which caused us to discard the possibility of enhancement of catalytic activity by some sort of van der Waals' type interaction seemed operative here, too, and this second possibility was also discounted.

If the mechanism proposed by Higuchi and Marcus (12) is correct, the data in Tables I and II are a source of serious concern. Even though the rate of reaction is almost independent of glycol concentration, there can be no doubt of continuing slight increases in rate. Inasmuch as the mechanism proposed requires a reaction between two ionic species bearing the same sign, the data in Tables I and II are in direct conflict with the requirements of the Bronsted-Christiansen-Scatchard equation (14). This expression requires a *decrease* in the rate of reaction between ions of like sign when the dielectric constant of the medium is lowered.[1] In the present study, however, the rate of reaction *increased* when the dielectric constant was lowered.

According to Amis (17) the rate of a reaction between a positively charged ion and a dipolar molecule should increase as the dielectric constant of the reaction medium is lowered. The expression derived by Amis also predicts that the relationship between rate and dielectric constant is not linear but falls off with succeeding decreases in dielectric constant. The data in Table II seem to be quite in line with these requirements and thus lend support to the postulation that the reaction might involve an ion and a dipole.

Because the evidence cited above can hardly be said to offer any rigorous support, a means was sought to provide additional evidence in favor of the postulation. Amis' equation may, for the limiting case of head-on approach of an ion to a dipolar molecule, be written

$$\ln k_{D = D} = \ln k_{D = \infty} + \frac{Ze\mu}{Dk_bTr^2}$$

where D is the dielectric constant of the medium, Ze is the charge on the ion, k_b is the Boltzmann gas constant, T the absolute temperature, and r is a measure of the closeness of the approach of the ion. According to this equation, when the reactants are a cation and a dipolar molecule, a plot of $\ln k$ *vs.* $1/D$ should yield a straight line with a positive slope. In order to see whether or not our data would fulfill this requirement, however, it was necessary to know the dielectric constants of our water-propylene glycol systems at the temperatures employed to effect degradation.

A search of the literature failed to reveal any information concerning the dielectric constants of water-propylene glycol mixtures. We were forced, therefore, to make certain approximations. We realize that our approach was arbitrary and that the values and the plots derived from them are either meaningless or, at best, open to serious question. We felt, however, that any clarification which would result might possibly offset the liabilities inherent in our approach.

The data of Akerlof (18) and calculations made using the constants given by him for glycerol and n-propanol, along with similar calculations for propylene glycol (16), indicated that, from 20 to 100°, the dielectric constant of propylene glycol is approximately the arithmetic mean of the dielectric constants of glycerol and n-propanol with a maximum deviation of 3%. Akerlof also shows that his expressions for the temperature dependencies of the dielectric constants of glycerol and n-propanol hold

[1] The dielectric constant of water through the temperature range employed in this study varied from 59.6 to 56.1 as calculated from the work of Malmberg and Maryott (15). The similar variation in the dielectric constant of propylene glycol is 21.2 to 19.8 as calculated from the data of Morgan and Yager (16). Naturally, an increase in the concentration of glycol results in an overall decrease in the dielectric constant of the reaction system. For purposes of comparison, the dielectric constants of water and propylene glycol at 20° are 80.1 and 32.0, respectively (15, 16).

for aqueous solutions of these two substances as well as for the pure materials In view of the approximate relationship between the dielectric constants of pure n propanol, glycerol, and propylene glycol, we have assumed a similar relationship to hold for aqueous solutions of the three organic solvents The values thus arrived at for the dielectric constants of water propylene glycol mixtures were applied to our data in an attempt to see if a plot of log k *vs* $1/D$ would aid in interpreting our data

Our plots were of the required slope and definitely linear in the region between 10 and 50% propylene glycol The plots did not, however, include the points at zero glycol concentration Although these plots did lend slight weight to the possibility that the reaction could be classified as of the ion dipole type, the slopes of the straight line portions were so small that it did not seem possible to place any great deal of faith in the results It appeared that some alteration in approach would be necessary to achieve plots more indicative of the applicability of the Amis equation

The apparently very small effect of glycol concentration in the range 10 to 50% glycol led us to assume that the glycol did not exert any effect such as summarized in Fig 2 It appeared possible that any influence being exerted by the glycol might be purely electrostatic in nature It has already been noted that when the rate equation may be written

$$k_H = K_{H^+} [H_2O]$$

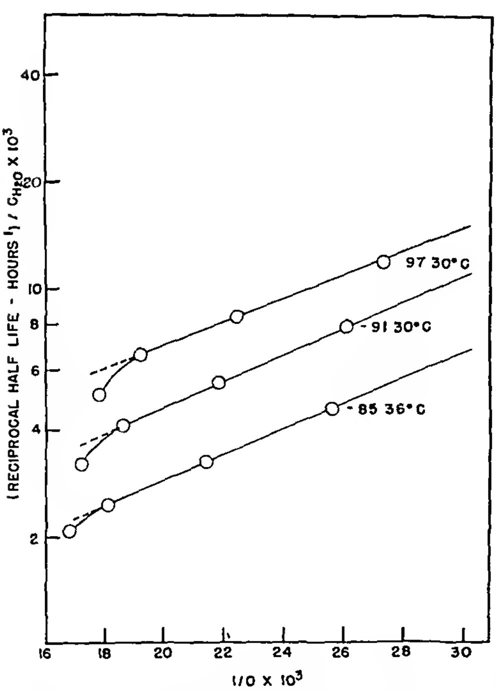
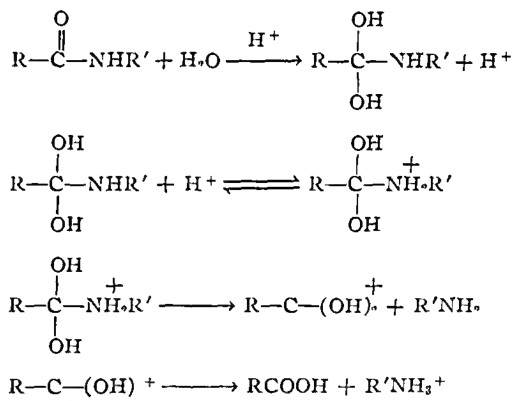


Fig 7—Plots indicating that our data for the reactions in 10, 30, and 50% (v/v) propylene glycol obey the requirements of the Amis equation for the effect of dielectric constant on the rate of a reaction between a cation and a dipolar molecule The reciprocal half lives were calculated from the data in Table II

it is unrealistic to ignore the decrease in water concentration if the effect of the organic solvent is purely electrostatic (19) A better test of the obedience of our data to the requirements of the Amis equation would be, therefore, to plot log (reciprocal half life)/C_{H_2O} as a function of $1/D$ [2] The results of these plots are shown in Fig 7

The magnitudes and the directions of the slopes in these plots do much to substantiate the thesis that the reaction studied involves an ion and a dipole When viewed in terms of the means used to estimate the dielectric constants, the almost strict adherence of the plots, in the range of 10 to 50% propylene glycol, to the Amis equation is almost incredible It should, therefore, be re emphasized that any attempt to represent these plots as having much quantitative significance would be foolhardy

Because of the degree of support in favor of the postulation of an ion dipole reaction, we have attempted to inspect the mechanism proposed by Higuchi and Marcus in the hope of finding some indication that the postulated ion dipole reaction does not represent either a "pipe dream" or a paradox If that mechanism is rewritten to conform with the mechanism proposed by Bender and Ginger (20) for the acid catalyzed hydrolysis of amides, we could write



If the first step above can be regarded to explain the apparent increase in the solubility of chloramphenicol with increasing concentrations of acid as resulting, not from the formation of an ionic substrate but rather as due to the formation of the more polar R C(OH) — NHR′, the overall reaction need no longer be considered to be between two ions of like sign Since the actual hydrogen ion catalyzed hydrolysis is dependent upon the second reaction, the dipole reaction may well be correct

It may be objected that the first reaction cannot be regarded alone but represents just a first step in a series of reactions leading to eventual cleavage of the C—N bond The experimental work showing an apparent increase in the solubility of chloramphenicol with increasing concentrations of acid was, however, carried out at 30° At this temperature the rate of hydrolysis of chloramphenicol (due to the second reaction) is almost negligible Under these

[2] Reciprocal half lives are related to the rate constants by the expression k = reciprocal half life × 0 693 for first order reactions

circumstances it is not at all surprising that only the fast reaction (the first) would receive attention.

The second reaction above is clearly of the ion-dipole type. If the mechanism is correct, our data are no longer paradoxical but conform directly with theoretical requirements. The above mechanism also does much to explain the fact that, in purely aqueous systems, the activation energy for the hydrogen ion catalyzed reaction is 5 Kcal./mole less than for the "water" reaction (12).

Thus far nothing has been said about the nonlinearity of the plots in Fig. 7 in the range 0 to 10% glycol. The data and supporting plots do little to illuminate this area. Landskroener and Laidler (21) have noted "kinetic anomalies" in the region of 10% by weight of the organic component. It is possible that these departures from linearity represent just such an anomaly. We are unprepared to venture any explanation until this region has been explored in greater detail. We do find one hopeful note in the plots of Fig. 7. It appears that the magnitude of departure from linearity decreases with a decrease in temperature. It is possible that the decreased effect of temperature on dielectric constant plays some part in this progression toward linearity. It is also possible, however, that the effect of propylene glycol in the 0–10% region is due to a specific solvent effect and is not amenable to treatment from a standpoint of electrostatic theory alone.

It has been suggested that the postulated ion-dipole reaction could receive additional support if the parameter r in the Amis equation were evaluated and found to be reasonable, i.e., if it were found to be of the correct order of magnitude for a molecular radius. Such an evaluation would require a knowledge or an approximation of the dipole moment of the substrate. Inasmuch as this value is not known and an approximation from the moments of other N-substituted amides would still leave us with the problem of further approximating the influence of the proton already present on the substrate, we feel that little is to be gained by such an effort.

TEMPERATURE DEPENDENCIES

The Arrhenius-type plots in Fig. 8 indicate the effect of temperature on the overall rates of degradation in the several solvent systems. The energies of activation, as determined from the slopes of these plots are 21.0, 20.5, and 20.0 Kcal./mole in the 10, 30, and 50% glycol solutions, respectively. For purposes of comparison, the activation energy in pure water is 19.5 Kcal./mole (12).

According to Amis (22), the most sensitive test for determining whether ion dipole reaction rates are influenced virtually exclusively by electrostatic effects lies in their obedience to the expression

$$\Delta E_c = \frac{69.1\, z u}{D_1 D_2 r^2}\, \Delta D$$

where E_c is the "coulombic activation energy," D_1 and D_2 are the dielectric constants of the media, z, u, and r have the same significance given previously. Our inability to evaluate the parameters r and u accurately prevents any precise testing of our data.

The direct relationship between activation energy and dielectric constant in systems containing from 10

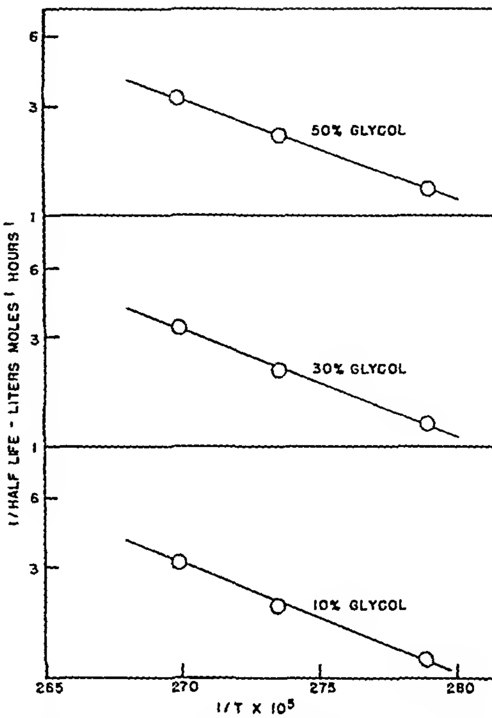


Fig. 8.—Arrhenius type plots showing the temperature dependencies of the reactions in 10, 30, and 50% propylene glycol. The activation energies as determined from the slopes of these plots are 21.0, 20.5, and 20.0 Kcal./mole for 10, 30, and 50% glycol, respectively.

to 50% glycol is in accord with apparent theoretical requirements. Amis and Holmes (23) state that for a reaction between a dipolar molecule and a cation, a decrease in dielectric constant should be accompanied by a decrease in activation energy. The value of 19.5 Kcal./mole for the reaction in purely aqueous systems is, of course, quite out of line with the rest of the values. Since there are a number (23–26) of recorded instances in which the direct relationship for reactions between cations and dipoles did not hold, it is possible that a direct relationship is not a rigid requirement of theory.

We are not, therefore, inclined to attach any great significance to the direction of change in activation energy with decreasing dielectric constant which we have found. It would, perhaps, be more logical to point out that our values for the activation energies are subject to an error of ±1 Kcal./mole. As a result, the differences noted above may be of doubtful significance.

PHARMACEUTICAL SIGNIFICANCE

The preceding data, figures, and discussion make it clear that it is unwise to make any blanket assumption that replacement of all or a portion of the water in a pharmaceutical preparation will enhance the stability of an active ingredient. It is equally clear that the use of nonaqueous solvents to achieve greater solubility introduces the possibility of decreasing the stability of a potentially active ingredient. In order to evaluate any contributions which might be

due to a nonaqueous solvent, prior kinetic studies in purely aqueous and in mixed solvent solutions are virtually mandatory. A knowledge of both the *apparent* and *operative* mechanisms is similarly desirable if stability is to be predicted with any accuracy.

The present study serves well to point up the necessity for proper interpretation of any stability studies. It might be assumed, for example, that the inclusion of chloramphenicol in acidic solutions, of which propylene glycol is a constituent, could lead only to decreased stability of the antibiotic. Such apprehension might be particularly severe if a comparison were made of the relative stabilities in 0 and 10% propylene glycol, omitting studies in solutions containing higher concentrations of the glycol. Since the function of propylene glycol in such a preparation would undoubtedly be to increase the solubility of the antibiotic and thus permit therapeutically effective doses, a potentially useful solvent might be discarded because of insufficient information and misinterpretation of the available data.

For one thing, the inclusion of 10% propylene glycol will not increase the rate of degradation of chloramphenicol by more than 10%. This is not, of course, an insignificant factor and, from this superficial viewpoint, one might understandably be loathe to investigate systems containing higher concentrations of propylene glycol. This reticence might well apply even when it became clear that only by increasing the glycol concentration to 50% could therapeutically effective concentrations be kept in solution. Had the region between 10 and 50% glycol been investigated, however, the relative independence of the rate of degradation on glycol concentration would have been established. At that point, perhaps, a decrease in the stability of the antibiotic by 10% might have represented a small price to pay for achieving solutions containing therapeutically effective amounts in reasonable dose volumes.

The value of studies such as the present one may be seen from still another angle. A formulator aware of the mechanism proposed for the hydrogen ion catalyzed degradation of chloramphenicol in purely aqueous systems might, conceivably, attempt to enhance the stability of the antibiotic in solution by effecting a decrease in dielectric constant. Such an effort would be quite logical according to the Bronsted-Christiansen-Scatchard expression. Had he, however, used propylene glycol to achieve this effect, the results of the present study clearly show that his efforts would have been in vain. It is obviously important to be aware of the mechanism which is operative as well as the one which is apparent.

We do not wish to suggest that a solution of chloramphenicol in perchloric acid, no matter how dilute the acid, represents a plausible dosage form. We do wish to point out, however, that the factors important here may be of equal importance in systems more plausible as dosage forms containing active ingredients less resistant to degradation.

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A Comparison of the Kinetics of the Acid Catalyzed Hydrolyses of Procainamide, Procaine, and Benzocaine*

By ARNOLD D. MARCUS† and STEPHEN BARON

The kinetics of the acid catalyzed hydrolyses of procaine and benzocaine have been found to be first order with respect to the esters and the rates linearly dependent upon acid concentration. The catalytic constants for the acid catalyzed hydrolysis of procaine have been evaluated and compared with those obtained for procainamide. The differences in magnitudes have been compared and an attempt is made to explain these differences. The energies of activation have been determined to be 16.8 and 18.6 kilocalories/mole for procaine and benzocaine, respectively. The fact that the activation energy for procaine is 3.8 kilocalories/mole higher than for procainamide disproves a previous postulation that, in acid systems, procainamide responds as an ester rather than as an amide. The kinetics and mechanisms of the acid catalyzed hydrolyses of procainamide, procaine, and benzocaine have been compared and analyzed from a standpoint of the activation energies, frequency factors, and entropies of activation. The apparently "unreasonable" values of the frequency factors and activation energies of procainamide and procaine have been examined in terms of both collision and transition state theories. It appears that when the reactions are regarded to involve ions of like sign, the relative and absolute magnitudes of the frequency factors and activation energies are not "unreasonable."

In a previous communication (1) it was reported that, contrary to apparent theoretical requirements, the activation energy for the acid catalyzed hydrolysis of procainamide is a surprisingly low 13.0 Kcal./mole. In an attempt to explain this apparent deviation from theory within the framework of apparently well-established mechanisms (2, 3), it was proposed that, in acid systems, procainamide responds as an ester rather than as an amide. Since no experimental evidence concerning the validity of this postulation was available, it was noted that similar studies on procaine (the ester) and benzocaine would be initiated.

The present communication reports the results of a study of the kinetics of the acid catalyzed hydrolyses of procaine and benzocaine. If it could be shown that the activation energy for procaine is 13.0 Kcal./mole or lower, the postulation that procainamide responds as an ester would receive considerable support. On the other hand, if it were found that the activation energy for the acid catalyzed hydrolysis of procaine exceeds 13 0 Kcal /mole, a revision in the proposed mechanism would be necessary. Although it was felt that a study of benzocaine would be of some significance in proving or disproving the postulation, its major function seemed to be in permitting some evaluation of the contributions of the β-diethylammonium function.

EXPERIMENTAL

Procaine.—The general procedure and analytical method of Higuchi, *et al* (4), were used throughout, with the following modifications: The withdrawn samples were diluted only tenfold at first with cold water. Aliquots of these dilutions were then diluted tenfold with 0 5 *M*, pH 9 5 carbonate-bicarbonate buffer just prior to spectrophotometric analysis The degradations were effected in 0.25 to 1 0*M* perchloric acid solutions

Benzocaine.—The same general experimental and analytical methods used for procaine were employed for benzocaine with the following modifications. Since benzocaine is poorly soluble in water, the 1% solutions of the ester were prepared with 0 5 *M* hydrochloric acid as the solvent Because the specific extinction coefficient and wavelength of maximum absorption of benzocaine differ from those of procaine, 5 0 ml , rather than 10 0 ml , portions of 1% solution were added to the reaction flask As a result of these modifications, the equation used to calculate the degree of hydrolysis must be rewritten as

$$\% \text{ residual ester} = 100 \times \frac{(k - k_{\text{PABA}})}{(k_{\text{benz}} - k_{\text{PABA}})}$$

where

k = the observed absorbance of the partly hydrolyzed sample diluted to 0 000500% with respect to original benzocaine, at 285 mμ

k_{PABA} = the absorbance of *p*-aminobenzoate ion equivalent to 0 00500% of benzocaine, at 285 mμ

k_{benz} = the absorbance of a 0 000500% solution of benzocaine at 285 mμ

All measurements were made at pH 9.5.

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EXPERIMENTAL RESULTS

Figures 1 and 2 show the acid catalyzed hydrolyses of procaine and benzocaine to be first order with respect to the substrates over a range of acid concentration and temperature. The expected linear relationship between rate of hydrolysis of procaine and acid concentration is evident from Fig. 3. The catalytic constants for the hydrogen ion have been evaluated from the slopes of these plots and are included in Table I. The previously determined (1) constants for procainamide are also included for purposes of comparison.

Figure 4 shows that the rate of hydrolysis of benzocaine at 97.30° is linear with acid concentration. Although we have not investigated the relationship at lower temperatures, it is reasonable to assume similar dependencies on the basis of other studies (5, 6).

Temperature Dependencies.—The activation energies for the two acid catalyzed reactions have been determined from the slopes of the Arrhenius type plots in Figs. 5 and 6. These are 16.8 and 18.6 Kcal./mole for procaine and benzocaine, respectively. Table II includes the first order rate constants, k, activation energies, ΔH_a, frequency factors, A, and entropies of activation, ΔS_a, for the acid catalyzed hydrolyses of procaine, procainamide, and benzocaine. The values for the latter two characteristics were calculated from the equation

$$k = \frac{RT}{Nh} e^{\Delta S_a/R} e^{-\Delta H_a/RT}$$

where

$$A = \frac{RT}{Nh} e^{\Delta S_a/R}$$

For the sake of uniformity and ease of comparison the data in Table II all refer to reactions in 0.5 M perchloric acid at 97.30° and k has the dimension seconds^{-1}.

TABLE I.—CATALYTIC CONSTANTS FOR THE HYDROGEN ION CATALYZED HYDROLYSES OF PROCAINE AND PROCAINAMIDE

T – °C	k_{H^+} Liters Moles^{-1} Hours^{-1}—Procaine	k_{H^+} Liters Moles^{-1} [a] Hours^{-1}—Procainamide
97.30	0.2775	0.0523
91.30	0.1975	0.0379
85.30	0.1265	0.0272

[a] Data of Marcus and Taraszka (1).

DISCUSSION OF THE EXPERIMENTAL RESULTS

Since the activation energy for the acid catalyzed hydrolysis of procaine is 3.8 Kcal./mole greater than for procainamide, we must conclude that the previously postulated mechanism for procainamide (1) is erroneous. A reconsideration of this mechanism will be presented in a subsequent section.

Benzocaine.—The value of 18.6 Kcal./mole for benzocaine is in general agreement with theoretical requirements in terms of substituent effects (5). The magnitude of the difference, however, seems small when regarded solely in terms of the electrophilic character of the p ammonium substituent. In 50% ethanol the activation energy for the acid

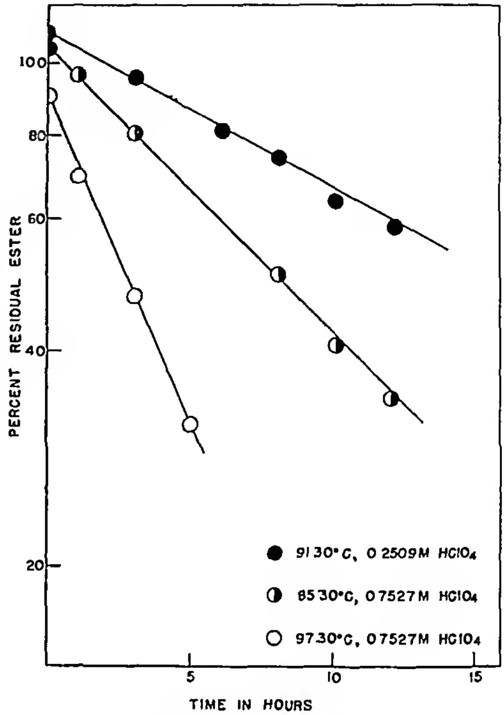

Fig. 1.—Plots showing the acid catalyzed hydrolysis of procaine to be first order with respect to the ester under a variety of experimental conditions.

TABLE II.—FIRST ORDER RATE CONSTANTS, ENERGIES OF ACTIVATION, FREQUENCY FACTORS, AND ENTROPIES OF ACTIVATION[a]

	k sec.$^{-1}$	ΔH_a Kcal./mole	A sec.$^{-1}$	ΔS_a Entropy Units
Procainamide[b]	7.26	13.0	3.43×10^{2}	−47.4
Procaine	38.5	16.0	3.14×10^{5}	−33.8
Benzocaine	140.0	18.6	1.32×10^{7}	−26.4

[a] All values refer to 0.5 M perchloric acid at 97.30°.
[b] Calculated from the data of Marcus and Taraszka (1).

catalyzed hydrolysis of ethyl p nitrobenzoate is, for example, 17.52 Kcal./mole (5). Since a p ammonium function is more electrophilic than a p nitro substituent, the value of 18.6 Kcal./mole for benzocaine must represent, in part, other than electrophilic effects. It would appear logical to assume that this value represents the net effect of electrophilic character and coulombic repulsion. The difference in solvent systems does, of course, play some part but the comparisons and conclusions remain valid.

Procaine Versus Benzocaine.—The preceding discussions and comparisons indicate that coulombic repulsion may be important in determining the activation energy of a reaction. It is, therefore, difficult to reconcile the activation energies for benzocaine and procaine with the expected coulombic repulsions. In the perchloric acid solutions employed in the present study, procaine is present as a doubly protonated species whereas benzocaine is only monoprotonated. Since acid catalyzed hydrolysis requires an additional protonation (2, 3), it seemed reasonable to ex-

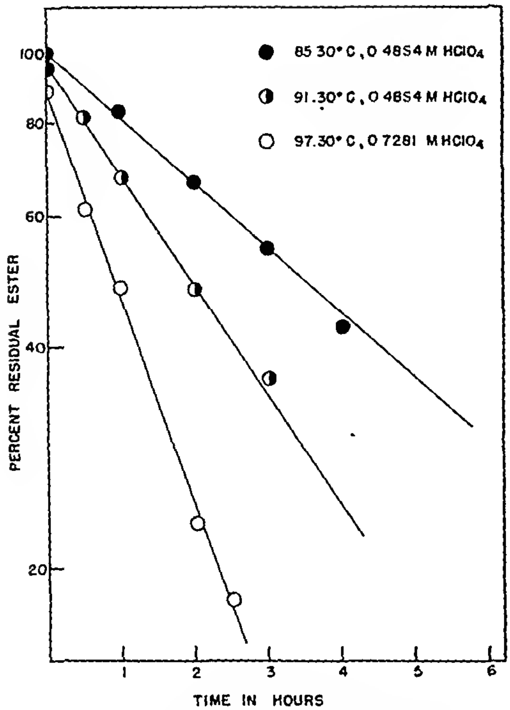

Fig. 2.—Plots showing the acid catalyzed hydrolysis of benzocaine to be first order with respect to benzocaine over a range of experimental conditions.

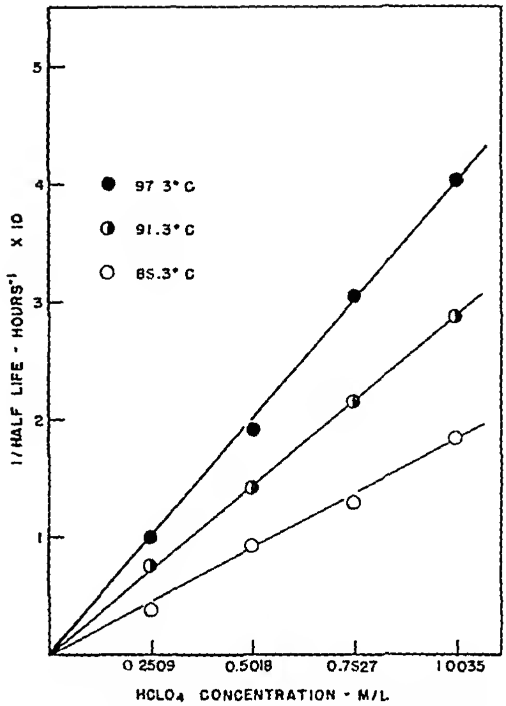

Fig. 3.—Plots showing the rate of the acid catalyzed hydrolysis of procaine to be linearly dependent upon the concentration of acid.

pect that the doubly protonated substrate would repel the catalytic species to the greater degree. The logical assumption which follows from this expectation would be that procaine should be associated with a higher activation energy than benzocaine.

Contrary to this assumption, the activation energy for the acid catalyzed hydrolysis is 1.8 Kcal./mole lower than for benzocaine. It is interesting to note that this is quite analogous to the acid catalyzed hydrolysis of procainamide in which every factor appeared to require an activation energy of perhaps 30 Kcal./mole. The experimentally determined activation energy is 13.0 Kcal./mole (1). Factors other than electrophilic substituents on the aromatic nuclei and coulombic repulsions are operative in these reactions. Indeed, it would seem that these additional factors are not only operative but that theirs are the predominating influences.

MECHANISMS OF THE REACTIONS

Influence on the Magnitudes of the Catalytic Constants.—Of all the data in Tables I and II, the differences in the magnitudes of the catalytic constants appear most amenable to treatment from straightforward considerations.

Since the present study apparently disproves the hypothesis that procainamide responds as an ester in acid systems, it must be assumed that the mechanism for the acid catalyzed hydrolysis of procainamide is of the usual type for benzamides. According to these apparently well established mechanisms (2, 3), acid catalyzed hydrolysis of amides proceeds through protonation of the amide nitrogen. It has been pointed out previously (1) that the β-diethyl-

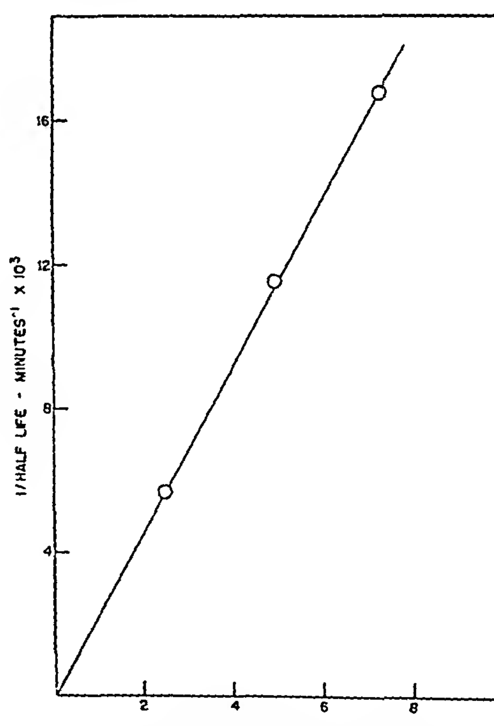

Fig. 4.—A plot establishing the direct relationship between the rate of the acid catalyzed hydrolysis of benzocaine and the concentration of acid at 97.30°.

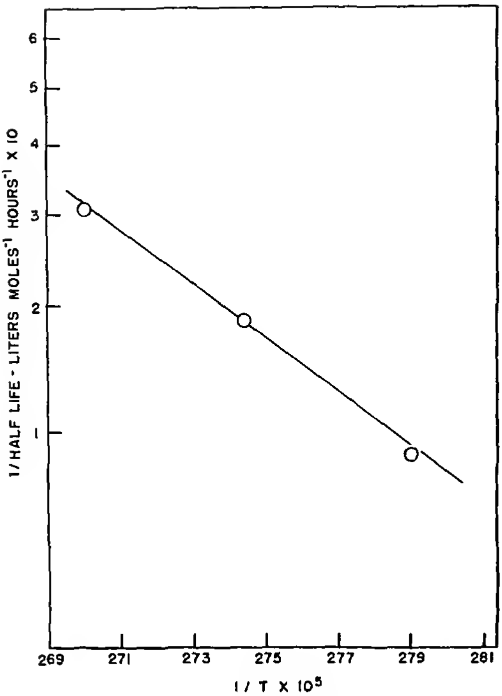

Fig. 5.—An Arrhenius-type plot showing the temperature dependency of the acid catalyzed hydrolysis of procaine. The activation energy for the reaction, as determined from the slope of this plot, is 16.8 Kcal./mole.

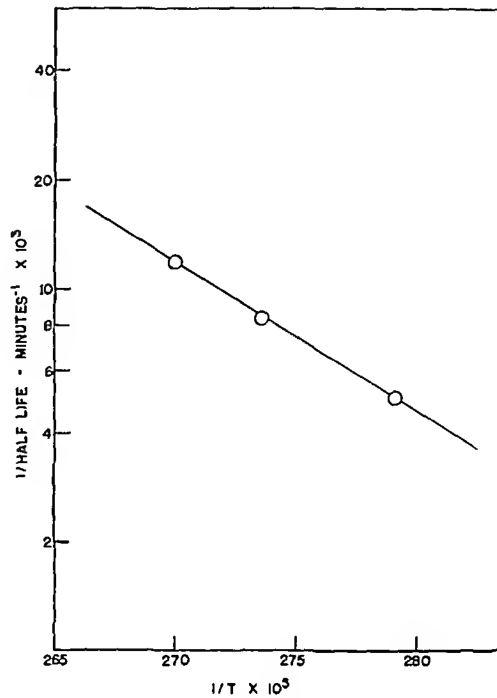

Fig. 6.—An Arrhenius-type plot showing the effect of temperature on the rate of the acid catalyzed hydrolysis of benzocaine. The activation energy as determined from the slope of the line is 18.6 Kcal./mole.

ammonium function is sufficiently electrophilic to markedly reduce the basicity of the amide nitrogen. As a result, protonation is more difficult and the efficacy of the catalytic species (H_3O^+) is decreased. This decrease is reflected in the magnitude of $k_H{}^+$. Since the acid catalyzed hydrolysis of esters also requires protonation (at the ester oxygen) we might expect $k_H{}^+$ to be almost the same for procaine and procainamide.

In the acid catalyzed hydrolysis of esters, however, even though protonation is important, it is much less important than in the hydrolysis of amides (2). On the other hand, in the acid catalyzed hydrolysis of an ester, attack by a water molecule at the carbonyl carbon is of much greater consequence than in amide hydrolysis (2). Inasmuch as the carbonyl carbon atom is much farther from the electrophilic β-diethylammonium substituent, the effect is materially reduced. In fact, the electrophilic character of the *p-ammonium function* would actually facilitate the acid catalyzed hydrolysis of an ester. Lastly, the effect of coulombic repulsion of the proton would be greater in the case of an amide than in the case of an ester. This results from the fact that protonation of the amide nitrogen, a site close to the formal positive charge, plays a much greater role in the hydrolysis of amides than in the hydrolysis of esters. There thus appear to be three different but related factors which account for the relative magnitudes of the catalytic constants.

Any rationalization or explanation of the values for the activation energies, frequency factors, or entropies of activation (Table II) presents a more difficult problem. When compared with the corresponding values for the acid catalyzed hydrolyses of ethyl *p*-nitrobenzoate or *p*-nitrobenzamide (2, 5) the frequency factor for procaine appears low by a factor of 10^3, and that of procainamide low by a factor of 10^8 or 10^9. Only benzocaine is associated with a frequency factor of "reasonable" magnitude. The large negative entropies of activation fall into a similar pattern and the activation energies are almost incredible. It is possible, however, that when viewed in a different light, the values for these reactions may appear more credible and reasonable.

Actual Nature of the Reactions.—By referring to a reaction between a proton and procainamide, procaine, or benzocaine, we leave the impression that the reaction involves an ion and a *dipolar molecule*. This is, of course, a false impression. The reactions are actually between ions of like sign. Since it is much easier to write "procaine" than β-diethylammoniumethyl *p*-ammoniumbenzoate, we shall retain the former designation. We wish to emphasize, however, that the substrate is a diprotonated species. The same cautions hold for procainamide and benzocaine (mono-protonated). Since the reactions are between ions of like sign, the values for the frequency factors and the entropies of activation may not be "out of line" and may even help explain the variations in the activation energies.

Procainamide

The frequency factor, A, may be regarded as approximately equivalent to the pZ term of the collision theory, and the steric factor, p, is associated

with the entropy term. It is, therefore, possible to consider the unusually low values of A and activation energy for procainamide from two viewpoints.

Collision Theory.—If a collision between two reactants of proper energy content is to result in a reaction, the colliding species must be properly oriented with respect to each other. Unless they are so oriented, the energy content of the reactants may be of little consequence. It has been found that in reactions between ions of like sign, p may have a value as low as 10^{-9} (7). In the case of procainamide, the two protons already present could be expected to exert a considerable repulsive effect when a third proton approaches. This effect would result in sufficient steric hindrance to almost demand a very low value for p and, therefore, of pZ or A.

When this effect is considered together with the reduced basicity of the amide nitrogen, it is not at all difficult to imagine that the third protonation will require the strictest orientation of the reactants. Furthermore, even though attack by a water molecule at the carbonyl carbon is not so important as protonation of the amide nitrogen, this attack must also occur. As a result, the reaction requires orientation, not only with respect to the ions, but with respect to a molecule of water as well. It would appear, therefore, that the rate of acid catalyzed hydrolysis of procainamide is considerably more dependent upon proper orientation of the reactants than upon their energy content at the time of collision.

The orientation requirements are so strict that only a few collisions can be effective. When, however, the third protonation does take place, the product is a triply charged ion. Not only is the new entity triply charged, but the charges are so close together that the new species will, figuratively speaking, tend to tear itself apart despite a low energy content.

Transition State Theory.—A similar conclusion may be reached if the reaction is considered in terms of a transition state or activated complex. When ions of the same sign react, the product is a more highly charged ion. Solvation of the activated complex would then require many more solvent molecules than the individual reactants. The consequent reduction in the freedom of the solvent would lead to a substantial decrease in entropy (a large negative entropy of activation) in forming the transition state. This would, in turn, require a low value for the frequency factor and, as above, the frequency factor rather than the energy content of the reactants would predominate in determining the rate of reaction.

If the above reasoning is correct, the low values for A and ΔH_a for the acid catalyzed hydrolysis of procainamide are not particularly "unreasonable" but might actually be expected. In this connection, it is interesting to note the work of Jellinek and Gordon who have shown the actual substrate in the acid catalyzed hydrolysis of nicotinamide to be a doubly charged cation (8). The frequency factors given by these authors at 10^4 or 10^5 lower than for the acid catalyzed hydrolysis of p-nitrobenzamide (2). Here, too, the importance of the entropy term appears to be considerable. It must be pointed out, however, that the activation energy for the acid catalyzed hydrolysis of nicotinamide is approximately 7 Kcal./mole greater than for procainamide.

Procaine

From the preceding discussion it would seem logical to expect that the same reasoning should hold true for procaine. In part, it does. The value of A for procaine appears, as noted previously, to be low by a factor of 10^3. It is, therefore, reasonable to assume that the entropy term (or steric factor) remains very important in determining the rate of acid catalyzed hydrolysis. Two aspects of this comparison remain, however, to be explained. First, the activation energy for procaine is 3.8 Kcal./mole greater than for procainamide and, secondly, the frequency factor is higher by a factor of 10^3. It would thus appear that proper orientation of the reactants or a large negative entropy of activation can supply only part of the answer.

The answer may lie in the essential difference between the acid catalyzed hydrolyses of benzoates and benzamides. Since the electrophilic β-diethylammonium substituent can be expected to produce the same *relative* decrease in the basicities of the amide nitrogen and the ester oxygen, it is possible that protonation of the ester oxygen (the weaker base) will require greater energy on the part of the reactants. This would then be reflected in the magnitude of the activation energy. Furthermore, the p-ammonium substituent on the aromatic nucleus of the acid moiety can be expected to facilitate attack by a water molecule at the carbonyl carbon atom (2). This ease of attack may then be reflected, not in the activation energy, but in a relaxation of the strictness of the orientation requirements. As a result, effective collisions could occur more frequently and this would, in turn, lead to a higher value for A (pZ). Inasmuch as attack at the carbonyl carbon is of greater importance in ester hydrolysis, the above explanation appears to offer a reasonable means of rationalizing the differences between procaine and procainamide.

Benzocaine

The comparatively "normal" value for A in the acid catalyzed hydrolysis of benzocaine indicates that the entropy term is not the major influence in determining the rate of hydrolysis. It would appear that the absence of the β-diethylammonium substituent results in a concomitant absence of any great steric hindrance. The difference in the entropies of activation for procaine and benzocaine is certainly evidence in this direction.

Except for the apparent coulombic repulsion which decreases the effect of the electrophilic p-ammonium substituent, the mechanism for the acid catalyzed hydrolysis of benzocaine is comparatively uncomplicated.

SUMMARY AND CONCLUSIONS

1. The rates of the acid catalyzed hydrolyses of procaine and benzocaine are first order with respect to the esters. There is a linear relationship between the rates of reaction and the concentration of acid.

2. The activation energies for these acid catalyzed hydrolyses are 16.8 and 18.6 Kcal./mole for procaine and benzocaine, respectively. The mag-

nitude of these activation energies indicates that a previously proposed mechanism for the acid catalyzed hydrolysis of procainamide was in error and this mechanism has been reconsidered.

3. Although the frequency factors and the entropies of activation for procaine and procainamide appear to be low by factors of 10^3 and 10^9, respectively, the values obtained appear more reasonable when examined in light of both the collision and transition state theories and their requirements for reactions between ions of like sign.

4. It is shown that an apparent deviation from theoretical requirements for a high activation energy may be explained in terms of a dominating influence of the entropy change or the "steric factor" upon the rate of reaction.

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The Relative Reliability of the Escape Reaction and Righting-Reflex Sleeping Times in the Mouse*

By DUANE G. WENZEL and HARBANS LAL†

The "escape reaction" method of measuring the end point of sleeping time in mice was compared to the "righting-reflex" method. The former was found to be more reliable, to have a more definite end point, to measure more effectively lower levels of CNS depression, and to measure cortical rather than midbrain reflexes.

NUMEROUS METHODS have been used to quantitate drug-induced sleep. Probably the righting reflex is the most widely-used end point. Although the relative importance of, and the interrelationships between the many variables involved in sleep are still poorly understood, it is associated with both a decrease in the level of consciousness and an overall diminution in somatic motor activity (1). The important criteria for sleep have been stated to be: loss of critical, as opposed to stereotyped reactivity to the environment; an increased threshold of general sensibility and reflex irritability; and the capacity to be aroused or brought back to the state of consciousness (2–4). The recovery of postural reflexes and diminution of the threshold of general sensibility and reflex irritability appear to be brought about through the mesodiencephalic system (4). This system operates in the absence or incapacitation of the cerebral cortex employing feedback circuits to the lower centers and the periphery. Such reactions have been described in the decorticated cat, dog (4), and monkey (5), and new-born infants or older anencephalic children (4). Thus, although the righting reflex is a possible end point of sleep, it gives no evidence of cortical involvement.

In one attempt to improve the end point of sleeping time a mouse is placed in a cell the dimensions of which are so slected that only a fully-conscious animal can climb out of the cell (6). Stimuli are provided by intermittent blasts of air. This approach appears to bracket the target of pharmacological sleep as the animal must not only regain all reflexes and irritability in order to perceive the stimuli, but must consciously consider means of escape. Additionally, the act of climbing from the cell requires the recovery of neuromuscular coordination. The escape reaction should theoretically yield therefore a relatively complete evaluation of drug-induced sleep.

The primary purpose of this study was to compare the experimental accuracy of the escape reaction method to that of the righting reflex in the evaluation of drug-induced sleeping time. A related objective was to demonstrate the role of the cerebral cortex in both methods.

EXPERIMENTAL

Sleeping times of male mice (18–25 Gm.) were determined with the commonly used experimental barbiturates, hexobarbital and pentobarbital. Six mice were used for each dose, 10 doses being employed for hexobarbital and 11 for pentobarbital with each of the three tests. In all tests sleep was

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TABLE I.—ANALYSIS OF VARIANCE

Source of Variation	Righting Reflex Degrees of Freedom	Righting Reflex Mean Square A[a]	Righting Reflex Mean Square B[b]	Escape Reaction Degrees of Freedom	Escape Reaction Mean Square
		Hexobarbital Sodium			
Between doses	9	2.1353	2.0095	9	0.3529
Linear regression	1	14.8980	14.5610	1	2.8773
Deviation from linearity	8	0.5400	0.4406	8	0.0373
Error	49	0.1008	0.1239	50	0.0158
Total	58			59	
		Pentobarbital Sodium			
Between doses	10	0.7512	0.6709	10	0.5533
Linear regression	1	6.8911	6.1422	1	5.1286
Deviation from linearity	9	0.9890	0.0630	9	0.0452
Error	55	0.0405	0.0428	53	0.0206
Total	65			63	

[a] First spontaneous righting. [b] Righting ability confirmed by repeated testing.

measured as the time interval between the intraperitoneal administration of the drug and the end point.

The escape reaction test was as previously described (6) except that six cells were employed simultaneously and an air reservoir of a heavy balloon was used. As some mice do not react to the air-blast stimulus, animals were first screened to eliminate those that did not come out of the cell within one hundred seconds. Two methods of recording the end point of the righting reflex were used. One employed the first spontaneous movement from the back to a normal position while in the second the animal was immediately returned to the back position. If the mouse remained in this position for at least thirty seconds, the awakening was regarded as a sleep movement and timing continued. The end point was two returns to the normal position each within the prescribed thirty-second interval.

Six mice which had demonstrated an escape reaction to the air blast were carefully decorticated and tested by both methods.

RESULTS AND DISCUSSION

The regression analyses of variance performed on the sleeping times are given in Table I from log-transformed data. It is seen that the two righting-reflex methods do not differ significantly. When the error of the initial spontaneous righting method, which is the smaller of the two, is compared to the error of the escape reaction it is found that the latter is significantly smaller. The level of significance is 0.1% in the case of hexobarbital and 1.0% for pentobarbital. When the data for each group of animals were individually examined, it was found that the lesser significance obtained with pentobarbital was the result of data from the higher doses employed. It would appear that the variance of the escape reaction method becomes greater with the increased duration of sleeping time. It was also observed that with the two smaller doses of hexobarbital (76 and 87 mg./Kg.) that only five out of twelve mice lost the righting reflex whereas the escape reaction test satisfactorily measured the shorter sleeping time.

The decorticated mice irreversibly lost the ability to perform the escape reaction but retained the righting reflex and other midbrain reflexes. This information, coupled with the longer sleeping times obtained with the escape reaction method, corroborates the initial assumption that this constitutes a more reliable criterion of cortical involvement than the righting reflex.

It may be concluded that the escape reaction test employed provides a more valid approximation of drug-induced sleeping time than the righting reflex method. Furthermore, it has an unmistakable end point, a smaller error, and effectively measures lower levels of CNS depression.

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Pharmaceutical Applications of the Sodium Salt of Carbopol 934*

By JAMES A. LEE and W. LEWIS NOBLES†

This study indicates that a number of medicinal agents can be satisfactorily suspended using the dry sodium salt of Carbopol 934. The emulsifying properties of this agent are also reported.

This study was undertaken in an effort to evaluate the suspending and emulsifying properties of the dry sodium salt of Carbopol 934,[1] (hereinafter designated as Carbopol). Of particular interest was the comparison of the behavior of this form of the agent with such properties *previously reported (1) for both the un*neutralized polymer and for preparations in which the acidic polymer was neutralized *in situ.*

Carbopol is a hydrophilic colloid originally designed as a thickening agent for various types of aqueous formulations. It is a high molecular weight polymer containing a large percentage of carboxyl groups. Essentially, it is a carboxylic vinyl polymer supplied as a finely divided white powder. It disperses readily in water to yield an acid solution of low viscosity.

CARBOPOL AS A SUSPENDING AGENT

Previous publications (1, 2) have indicated methods by which the agent may be most easily dispersed and neutralized to serve as a suspending agent and emulsion stabilizer That it can function effectively as both a primary emulsifier and emulsion stabilizer is a tribute to the unique gel-forming properties of this material Once the desired agent has been dispersed, coalescence is effectively prevented by electrical charge repulsions within the molecule The mechanisms responsible for the very high viscosities obtained are solvation and electrical charge repulsions within the molecule. Unfortunately, however, because of the latter effect, ionic materials added to a Carbopol-thickened system supply cations which are attracted to and surround the negative charges of the molecule The rigid electrical repulsion within the molecule is thereby lessened and the result is a marked lowering of viscosity unless the concentration of Carbopol is increased to offset the loss of optimum viscosity produced by the cations (4)

Carbopol salts are excellent suspending agents for many solids dispersed in aqueous systems Satisfactory dispersions of calamine, neocalamine, and a kaolin-pectin combination were previously reported Other workers (1, 3), however, had reported that certain multivalent metallic ions were poorly suspended It had been reported (3) that cosmetic and pharmaceutical applications of this agent had been hindered by the reaction of the multivalent metallic ions to form insoluble salts of Carbopol, resulting in unsightly, coarse preparations Some of these findings were in apparent conflict with our earlier observations relative to calamine, neocalamine, and zinc oxide (2) Nevertheless, on reexamination, our earlier findings were confirmed. During the past year, Cohen (3) presented a theory of salt formation which may serve satisfactorily as a basis for the interpretation of the results obtained with such substances The following equations thus are suggested as possible explanations of some of the difficulties previously encountered in the use of multivalent metallic ions with Carbopol:

case 1 (acid condition).

$$\begin{array}{l} \text{—COOH} \\ \text{—COOH}\ \oplus \\ \text{—COO}^{\ominus}\text{H}\ \oplus \end{array}\quad \begin{array}{l} \text{—COO}^{\ominus}\text{H}^{\oplus} \\ \text{—COOH} \\ \text{—COOH} \end{array} + 2\text{ZnO} \xrightarrow[\text{H}_2\text{O}]{[\text{H}^+]}$$

$$\begin{array}{ll} \text{—COO—Zn—} & \text{OOC—} \\ \text{—COOH} & \text{HOOC—} \\ \text{—COO—Zn—} & \text{OOC—} \end{array} + 2\text{H}_2\text{O}$$

(Cheesy precipitate)

case 2 (neutralized condition).

$$\begin{array}{l} \text{—COO}^{\ominus}\text{Na}^{\oplus} \\ \text{—COO}^{\ominus}\text{Na}^{\oplus} \\ \text{—COO}^{\ominus}\text{Na}^{\oplus} \end{array} + \begin{array}{l} \text{—COO}^{\ominus}\text{Na}^{\oplus} \\ \text{—COC}^{\ominus}\text{Na}^{\oplus} \\ \text{—COO}^{\ominus}\text{Na}^{\oplus} \end{array} + 2\text{ZnO} \xrightarrow[\text{H}_2\text{O}]{[\text{OH}^-]}$$

$$\begin{array}{l} \qquad\qquad\text{Na}^{\oplus} \\ \text{—COO—ZnOH} \\ \text{—COO}^{\ominus}\text{Na}^{\oplus} \\ \text{—COO}^{\ominus}\text{Na}^{\oplus} \end{array} + \begin{array}{r} \\ \text{Na}^{\oplus}{}^{\ominus}\text{OOC—} \\ \text{Na}^{\oplus}{}^{\ominus}\text{OOC—} \\ \text{Na—O—Zn—OOC—} \end{array} + [\text{OH}^-]$$

(Smooth dispersion)

While it is readily admitted that these equations are hypothetical in nature, they appear to interpret, fairly accurately, the results obtained with the use of Carbopol in such formulations In case 1, the formation of a cheesy precipitate with no viscosity in the liquid phase tends to support the concept of the formation of —COO—Zn—OOC groups upon the addition of zinc oxide to the acidic polymer In case 2, there is no direct evidence to support the conjecture that the —COO—Zn—OH group is actually present Brown and Duke (5), however, suggest the presence of such a linkage as being involved in the vulcanization of carboxylic rubber with zinc oxide Furthermore, this type of grouping is analogous to basic zinc acetate, $CH_3COO—Zn—OH$ In the presence of the sodium ion there is also the possibility of —COO—Zn—ONa groups being present

When the metallic oxide is added to a nonneutralized dispersion of Carbopol, an unsatisfactory dispersion of agglomerated particles may result, as suggested in case 1. If, however, the

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[1] A product of B F. Goodrich Chemical Co

Carbopol is neutralized to a pH of 7 or higher, prior to the addition of such agents, a smooth, stable dispersion may be obtained.

The following data (3) suggest that the poor suspending property of Carbopol in the presence of zinc oxide is due to the neutralization of this agent by the zinc oxide: (*a*) The pH of a 1% dispersion of zinc oxide in water is 6.9. (Taken alone this would not predict a basic reaction.) (*b*) The pH of a 1% dispersion of Carbopol 934 in water is 3.1, but (*c*) when 1.65 Gm. zinc oxide is added to 300 Gm. of a 1.0% Carbopol dispersion (equimolar amount of zinc oxide and Carbopol) and mixed in a Waring Blendor for thirty minutes, a grainy precipitate results, and the pH is raised to 6.3. This is positive proof of the zinc oxide neutralization of the carboxylic acid groups on the Carbopol and, in conjunction with the formation of a precipitate, strongly indicated the equation in case 1 (*d*) If sodium hydroxide solution is added to the precipitated formulation resulting in (*c*), and mixed for a very long time, the precipitate will disappear and the viscosity of the liquid phase will eventually increase greatly, indicating the disappearance of the —Zn— crosslinks in a manner as postulated in case 2. The following data demonstrate this effect:

In the course of the present preparative work, the dry sodium salt of Carbopol was utilized. This was prepared as reported elsewhere (6). By this method approximately 0.4 Gm. sodium hydroxide is used per gram of Carbopol. This ratio of alkali/Carbopol will produce a mucilage possessing a pH range of 7–7.6.

The results of this study are summarized in Table I. They may be compared with the results reported by Skauen and his co-workers (1).

The dry sodium salt of Carbopol works well in this type of application. This agent produces a satisfactory suspension and obviates the necessity of using any previously prepared solutions or the weighing of small quantities of the neutralization agent each time. The substances to be suspended can be dry blended (in the present work this was done on small runs in a mortar and pestle and on larger runs with the use of the Patterson-Kelley yoke type blender) with the sodium salt and then water may be added with trituration to give the desired volume.

The second phase of this study dealt with the use of Carbopol as a primary emulsifier and/or an emulsion stabilizer. This agent has been recommended and has found commercial application as an emulsion stabilizer. Skauen and his associates (1)

10% NaOH Added to 300 Gm. of Resulting Formulation in (*c*), ml.	Total Time Mixed in Waring Blendor at Full Speed, min.	pH	Brookfield 20 r. p. m. Viscosity	Comments
5.0	44	9.8	0	Ppt. much finer
13.0	116	11.5	0	Ppt. much finer
13.0	127	10.8	19,120	Smooth liquid phase, thick by paper test, no precipitation or agglomeration
	Sample aged 48 hours, min.			
15.0	127	9.8	17,800	Smooth, as above
15.0	134	11.4	8,000	Smooth, as above
10% acetic acid added	...	6.2	0	Grainy ppt. reforms, no viscosity

Thus, a good suspension can be formed by adding zinc oxide to acidic Carbopol followed by sodium hydroxide addition, but a tremendous amount of mixing must be used to eliminate the —COO—Zn—OOC— groups, once they are formed. The degree of mixing required is completely impractical and would appear to be of academic interest only.

(*e*) On the other hand, the use of preneutralized Carbopol mucilage eliminates the formation of precipitate (—COO—Zn—OOC—) as occurred in (*c*) and avoids the necessity of the tremendous amount of mixing employed in (*d*)

This may be illustrated by the following results utilizing the same ingredients:

	pH	Viscosity, c.p.s.
300 Gm. of a 1% Carbopol dispersion plus 10 ml. 10% NaOH	7.1	42,500
1.65 Gm. of zinc oxide added with three minutes mixing on Waring Blendor	8.5	34,200 (smooth, good)
10% additional zinc oxide added to formulation	8.6	36,500 (smooth, good)
Aged twenty-four hours	8.6	46,000 (smooth, good)

reported its use as an emulsifying agent when used with such substances as liquid petrolatum, cod liver oil, cotton seed oil, and turpentine oil.

When this agent is used as an emulsion stabilizer in a system involving other emulsifying agents, excessive amounts of Carbopol are necessary due to the ionic deswelling produced by the sodium ion, if the other agent be sodium stearate, sodium oleate, or a similar substance. Such emulsions are easily prepared by either the "dry" or "wet" gum method. To prepare an emulsion by the dry gum method, the oil was placed in a dry mortar and the desired quantity of the sodium salts was thoroughly incorporated. Water was then added, in portions; emulsification taking place while triturating. The appearance of the preparation improved considerably after it had been passed through a hand homogenizer several times.

To utilize the wet gum method, a mucilage was prepared from the dry sodium salt by the addition of water with constant agitation. The oil was added, in portions, with constant trituration. In this case also, the use of the hand homogenizer greatly improved the appearance of the product.

These emulsions may be more conveniently pre-

TABLE I.—SUSPENSIONS PREPARED USING DRY SODIUM SALT OF CARBOPOL 934 (OBSERVATIONS MADE AFTER NINETY-SIX HOURS)

Substances Used	Carbopol Salt 0.25%	0.5%	0.75%
Oil of pine tar, 5%, triethanolamine, 1.5%	Slight separation Shakes in well	No separation	
Coal tar, 5%	5% separation, good	No separation, too thick	
Ichthammol, 10%, glycerin, 3%	No separation, good		
Sulfur, 5%, glycerin, 3%	5% separation, good	No separation, good	
Starch, 10%, glycerin, 3%	40% separation, poor	No separation, good	
Benzocaine, 5%, neocalamine, 16%, glycerin, 3%		No separation, good	
Bismuth subcarbonate, 5%, glycerin, 3%	Slight separation, good	No separation, good	
Zinc oxide, 10%, glycerin, 3%		30% separation, poor	0.65% Carbopol salt, slight separation, good
Aspirin, 8%		Granular separation	Poor
Calamine, 8%, Zinc oxide, 8%, Glycerin, 4%	Granular, poor	0.65% Carbopol salt, no separation, good	

TABLE II.—EMULSIONS USING DRY SODIUM SALT OF CARBOPOL (OBSERVATIONS MADE AFTER ONE HUNDRED TWENTY HOURS)

	Percentages of Carbopol Salt Used 0.15	0.25	0.5	1	2
Liquid petrolatum, 50%		No separation		No separation	
Cod liver oil, 50%	2% separation	No separation			
Benzyl benzoate, 25%		20% creaming	15% creaming	No separation	
Cottonseed oil, 50%		35% separation	30% separation	No separation, too thick	
Turpentine oil, 15%				No separation	No separation

pared in a mixer (such as a Waring Blendor) by adding the oil and mucilage followed by high speed agitation.

The results of our studies, utilizing the dry sodium salt, are presented in Table II.

The advantage and speed of the present method are emphasized as being more desirable than other methods (1) which require the use of a solution of the neutralizing agent of the desired percentage strength or the weighing, each time, of the proper quantity of neutralizer.

SUMMARY

Satisfactory suspensions and emulsions of a number of commonly employed medicinals were prepared with the use of the dry sodium salt of Carbopol. In general, 0.5% of the dry sodium salt appears to be the optimum concentration for suspending purposes.

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Sterility of Antibiotic Ophthalmic Ointment*

By FRANCES W. BOWMAN and SIMON HOLDOWSKY

A survey was conducted to determine the sterility of antibiotic ophthalmic ointments. Forty-six samples representing nineteen batches were tested. Only 10 per cent of the batches were found contaminated, in contrast to approximately six times that percentage reported by other investigators.

THIS STUDY was prompted by the recent findings of Vander Wyk and Granston (1) that of twenty-eight samples of antibiotic ophthalmic ointments tested, seventeen were nonsterile. While the antibiotic regulations[1] governing the certification of such preparations do not require sterility testing, the Food and Drug Administration does insist that the *manufacturing facilities* be capable of producing essentially sterile products. Under the certification program (2), antibiotic ointments were tested for microorganism count from 1947 to 1950. During that time it was found that the incidence of contamination in ointments was very low. This finding resulted in a recommendation for discontinuance of such tests, and they were deleted from the antibiotic regulations in 1950 (3).

The method employed by Vander Wyk and Granston (1) differed from the former official method (2), and appeared to offer certain advantages in the efficiency of organism recovery. The old method consisted simply of squeezing approximately 0.1 to 0.5 Gm. of ointment on to the surface of 20 ml. of nutrient agar in a Petri dish. After the ointment was spread evenly over the agar with a sterile glass rod, the Petri dish was incubated at 37° for forty-eight hours. From the number of colonies appearing on the plate, the number of viable microorganisms per gram of ointment was calculated. The method of Vander Wyk and Granston introduces a dispersion step in which the ointment is shaken for one hour at room temperature with sterile glass beads in 25 ml. of distilled water. Three 1.0-ml. aliquots are then placed in Petri dishes, mixed with melted blood agar, and incubated at 37° for twenty-four hours.

EXPERIMENTAL

Forty-six commercial tubes of antibiotic ophthalmic ointment were tested by the procedure of Vander Wyk and Granston (1). These represented 19 batches from 10 manufacturers. All tubes were labeled to contain $^1/_8$ of an ounce of ointment.

Results.—As shown in Table I, contamination was detected in only two batches, both from the same manufacturer. In one of the batches, one tube tested was sterile while the other was not. In the other contaminated batch, six tubes were tested and all were found to be nonsterile. Both bacteria and molds were observed in each case. That these findings were not due to chance contamination is supported by negative findings with nine other batches, seven in duplicate, one in sextuplicate, and another with eight replicates.

TABLE I.—STERILITY TESTS ON ANTIBIOTIC OPHTHALMIC OINTMENT

Type of Ointment	Organisms/ Gm.
Penicillin G, 100,000 units/Gm.	
Batch 1 (1 tube)	0
Batch 2 (1 tube)	0
Batch 3 (2 tubes)	0
Penicillin G, 10,000 units/Gm. (1 Batch, 2 tubes)	0
Penicillin G, 1,000 units/Gm.	
Batch 1 (2 tubes)	0
Batch 2 (2 tubes)	0
Bacitracin, 500 units/Gm.	
Batch 1 (6 tubes)	0
Batch 2 (8 tubes)	0
Chloramphenicol, 1%	
Batch 1 (1 tube)	0
Batch 2 (1 tube)	0
Chlortetracycline Hydrochloride, 1%	
Batch 1 (1 tube)	0
Bacitracin, 500 units/Gm., and Neomycin Sulfate, 5 mg./Gm.	
Batch 1 (2 tubes)	0
Bacitracin, 500 units/Gm., and Polymyxin B Sulfate, 10,000 units/Gm.	
Batch 1—tube 1	0
Batch 1—tube 2	79
Batch 2—tube 1	30
Batch 2—tube 2	84
Batch 2—tube 3	28
Batch 2—tube 4	22
Batch 2—tube 5	22
Batch 2—tube 6	42
Bacitracin, 400 units/Gm., Neomycin Sulfate, 5 mg./Gm., and Polymyxin B Sulfate, 5,000 units/Gm.	
Batch 1 (2 tubes)	0
Batch 2 (2 tubes)	0
Batch 3 (2 tubes)	0
Bacitracin, 500 units/Gm., Neomycin Sulfate, 5 mg./Gm., and Polymyxin B Sulfate, 5,000 units/Gm.	
Batch 1 (2 tubes)	0
Bacitracin, 500 units/Gm., Neomycin Sulfate, 3 mg./Gm., and Polymyxin B Sulfate, 10,000 units/Gm.	
Batch 1 (1 tube)	0

DISCUSSION

Our findings do not fully corroborate those of Vander Wyk and Granston. Assuming that each of

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[1] The antibiotic regulations are issued under section 507 of the Federal Food, Drug, and Cosmetic Act

the 28 samples tested by them represented a different batch, only 39% of the antibiotic ophthalmic ointments were sterile. Of the 19 batches tested by us, about 90% were sterile, a finding which agrees essentially both with four years' experience in testing antibiotic ointments from 1947 to 1950 and with results of a survey of ointments containing sulfonamides (4).

SUMMARY

Forty-six samples of antibiotic ophthalmic ointments were tested for sterility. Of the 19 batches represented, 90 per cent were sterile. This would indicate that the status of these ointments respecting the degree and incidence of contamination is more satisfactory than would be gathered from the results obtained by Vander Wyk and Granston.

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Influence of Dissolution Rate and Surface on Tetracycline Absorption*

By EINO NELSON

With the Technical Assistance of YOKO YUZURIHA

Tetracycline absorption was studied by means of excretion rate measurements in humans after 200-mg. doses of tetracycline or its salts. A direct correlation was found between excretion rate at one, two, and three hours and *in vitro* solution rate of the drugs when the dose was given in the form of two 0.95-cm. diameter pellets. Differences in rate were less noticeable when the dose given consisted of particles having an average diameter of 100 microns. The absorption of tetracycline, tetracycline phenolsulfonphthaleinate, and tetracycline sodium hexametaphosphate complex was rate-limited by *in vivo* solution rate to some degree. Tetracycline hydrochloride absorption was rate-limited by the absorption process itself. The bases for these correlations were discussed. The mathematics of urinary excretion was considered to the extent of pointing out the relationship between excretion rate and blood level.

THE ABSORPTION of tetracycline after oral ingestion by humans has recently been the subject of many investigations (1–7). These studies have dealt, for the most part, with serum levels obtainable when tetracycline or its salts were taken with or without additives by the oral route. With the exception of the study in which rather marked depression in serum levels was obtained when tetracycline hydrochloride was given with dicalcium phosphate (4), no clear picture has emerged to explain what factor or factors control the absorption of tetracycline.

In all of the investigations cited above, no attempts were apparently made to control or characterize the physical form of tetracycline in the capsules used as the dosage form. On the basis of particle size alone, which determines initial surface presented to dissolution mediums after ingestion, differences in blood levels should reasonably be expected in accordance with the results found in studies on sulfonamides (8, 9). In these two studies, micronized powdered sulfonamides gave higher levels than microcrystals. Another consideration in absorbability relates to the intrinsic solution rate properties of the same base compound in the form of different salts. This consideration, as well as the expected effect of other factors influencing solution rate of drugs at absorption sites, has been discussed recently (10–12).

It is only reasonable to expect that physical and physical-chemical factors should influence tetracycline absorption when the nature of the absorption process itself is considered. According to a recent report (13), tetracycline appears to be absorbed by free diffusion. An "absorption enhancement" due to additives or a particular salt used could not occur in this case since this would require involvement with a transport mechanism in absorption. Even if a transport mechanism were involved in tetracycline absorption, availability of drug in solution at absorption sites would still be required and it is conceivable that the rate-limiting step could be the rate of the solid to solution step. With absorption by free diffusion, the overall absorption process is even more susceptible to being controlled in rate by the rate of dissolution of drug at

* Received June 11, 1958, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

absorption sites because of the concentration dependency of this process.

In comparative studies of the type which have been made on tetracycline, the considerations of effect of chemical nature of drug on absorbability are not pertinent since, regardless of the salt or mixture used, the same drug is involved in absorption. The dependency of absorption on pK and fat solubility of drugs is discussed (14–16) and the hypothesis advanced is involved in tetracycline absorption as such.

Another factor could have an influence on the overall absorption process of tetracycline. This is concerned with presence of materials in gastrointestinal tract capable of binding tetracycline and reducing its availability for absorption. Possibly, additives given with tetracycline could inhibit or prevent this occurrence, but no judgment of effectiveness is possible until the influence of the physical and chemical factors also involved is controlled and reproduced for successive trials

The present report gives the results of excretion rate studies on tetracycline given in several different chemical and physical forms and discusses correlations that exist between these results and the physical-chemical property of solution rate and factors affecting this rate. This report also discusses the nature of the mathematical relationship between excretion rate and blood level.

EXPERIMENTAL

Materials and Assay.—The substances used in tests included tetracycline hydrochloride, tetracycline sodium hexametaphosphate complex, and tetracycline [1] Some tests were also made using the phenolsulfonphthalein salt of tetracycline [2] The activity of these preparations, when assayed by the cylinder-plate method (17) using *Bacillus cereus* var. *mycoides* as the test organism, is shown in Table I. This was also the assay procedure for urine samples Urine samples were kept at 1° until diluted with 0.1 *M* monobasic potassium phosphate. Dilutions were usually made within twenty-four hours Diluted samples were also stored at 1° and usually assayed within three days or less Blank urine samples were treated and assayed in the same manner.

Subjects.—The test panel consisted of ten adult humans all in apparent normal health.[3] The ages and weights of the subjects are given in the first entry in Table II. The ten subjects were not all used in every cross comparison. Their participation in tests is indicated by the entries in Tables II and III.

Conduction of in Vivo Tests.—The dosage forms were taken on either a fasting stomach in the morning or at least one hour after a toast and coffee breakfast according to the individual preference of the subjects No food was taken for at least one hour after ingestion of the dosage form. Each subject was instructed to drink water in an amount sufficient to allow him to collect urine samples at least at one, two, four, and eight hours The volumes voided were noted and an aliquot taken for assay One week usually elapsed between tests

Preparation of Dosage Forms.—Two types of dosage forms were used In the first type, two pellets of each material 0 95 cm in diameter and about 0 15 cm thick, pressed at about 1000 Kg /sq cm and containing 200 ± 10 mg of tetracycline hydrochloride activity were packed in 000 capsules with 200 mg sodium bicarbonate interspersed between the pellets and in the ends of the capsule The pressings were made by a technique previously described (11). In the second type of dosage form, small particles made by grinding pellets of the type described above and sieving to collect the 115/200 (Tyler series) mesh fraction were packed in 00 capsules after loosely mixing with 200 mg sodium bicarbonate Each capsule contained 200 ± 1 mg. tetracycline hydrochloride equivalent Prior to placing pellets in capsules or grinding to prepare small particles, the lubricant remaining on their outer surfaces as a result of the compression process was removed by rubbing with a fine grade emery cloth. The dosage forms were taken within a day of preparation to avoid reaction between the salts and sodium bicarbonate.

The pellets formed by the process described were quite hard Soaking in water at room temperature did not cause excessive disintegration in times up to two hours.

In Vitro Solution Rate Tests.—The same materials used in *in vivo* studies were compressed into pellets 1 3 cm in diameter and about 0 3 cm thick by the technique and at the same pressure used in preparing the smaller pellets for *in vivo* tests. The dissolution rates of these pellets at 37 ± 2° in simulated gastric fluid (18) and in two simulated intestinal fluids (19) were measured by a free convection method (11). The simulated intestinal fluids were the neutral and alkaline solutions described in the cited report (19).

RESULTS AND DISCUSSION

Excretion Data.—Tables II and III summarize the excretion data collected in all tests including volumes of urine collected Since it was not practical always to collect urine specimens at the even hour headings shown in these tables, the tabulated data represent values read at the stated intervals from individual plots of cumulative amounts excreted with time The urine volume data for table entry were obtained in the same way except that linear interpolation was used to find volumes at even hours With tetracycline excretion data, interpolations were made on the best line through

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[1] These pure materials were supplied at various times by the Bristol Laboratories, E R Squibb and Sons, and the Chas Pfizer Co The author is grateful for this assistance The tetracycline sodium hexametaphosphate complex was supplied by the Bristol Laboratories

[2] Made by mixing aqueous solutions of tetracycline hydrochloride and the monosodium salt of phenolsulfonphthalein in stoichiometric quantities, collecting the precipitate, washing with several volumes of cold water, and drying over sulfuric acid in partial vacuum at room temperature The dried material was used without further purification in all tests This compound will be referred to as tetracycline PSP

[3] This work was facilitated by a most cooperative test panel to whom the author is indebted.

the experimental points This procedure was desirable since the amount excreted at various fixed times was necessary for interpretation of results

Interpretation of Excretion Data.—The potential of excretion data to evaluate drug absorption does not appear to be generally recognized in spite of a considerable amount of data in the literature describing drug absorption, distribution, and excretion kinetics validating its use (see, for example, references 20–23) For those drugs that are eliminated in whole or in part unchanged in the urine and that show an exponential decay in concentration in the blood stream after drug in blood is in equilibrium with the other fluids of distribution, excretion rate is directly proportional to the amount of drug in the blood. Hence,

$$\frac{da_e}{dt} = ka_b \qquad \text{(Eq. 1)}$$

where a_e is the amount excreted in time t, a_b is the amount of drug in the blood, and k is a constant characteristic for a given drug and incorporates the specific elimination rate constant and the fraction of drug eliminated via the kidney (25) Equation 1 assumes that clearance does not change with urine elimination rate.

In some of the work described in this paper, it was not necessary to know the value of the constant k since it canceled in any comparison which was a ratio of excretion rates Thus, when mean excretion rates at a given time from two preparations were compared, their ratio was the ratio of blood levels at the same time.

TABLE I —ACTIVITY OF PREPARATIONS USED IN STUDIES[a]

Preparation	Theoretical	Found
Tetracycline HCl	100	100
Tetracycline sodium hexametaphosphate complex		76
Tetracycline PSP	60	63
Tetracycline	108	90

[a] Expressed in terms of per cent of activity of a sample of commercial tetracycline hydrochloride taken as standard

TABLE II —EXCRETION DATA FROM SINGLE 200-MG DOSES OF TETRACYCLINE IN PELLET FORM[a]

Cumulative mg of Tetracycline and ml Urine Excreted to Various Times in Hours

Subject[b]	mg as Hydrochloride 1 0	2 0	3 0	4 0	6 0	8 0	Urine Volume, ml 1 0	2 0	3 0	4 0	6 0	8 0
Tetracycline PSP												
E (39–77)	1 0	5 6	10 0	13 1	18 1	22 0	740	1080	1240	1350	1470	1600
S (33–57)	0 5	3 8	8 6	14 0	19 3	21.5	60	120	190	270	430	520
B (28–82)	0 4	3 0	5 8	7 9	11 3	14 2	30	60	90	120	200	280
T (23–75)	1 0	4 4	8 6	11 7	16 5	20 1	60	110	160	200	220	340
N (21–77)	0	4 0	13 5	21 3	34 5	45 6	80	200	270	340	440	540
H (26–73)	0 5	2 5	5 9	9 8	14 7	17 8	50	170	450	740	830	930
L (30–59)	0 2	2 1	5 2	8 8	12 6	14 2	50	200	370	540	820	1070
A (24–84)	0 2	3 8	8 5	12 4	18 3	23 9	40	120	200	290	520	750
J (23–61)	1 1	4 2	6 2	7 6	9 0	9 5	150	460	560	660	770	870
R (26–84)	0 3	1 6	4 7	8 0	12 1	13 5	40	60	80	110	140	180
Mean	0 5	3 5	7 7	11 5	16 6	20 2	130	258	361	462	584	708
Tetracycline Sodium Hexametaphosphate Complex												
E	0 5	4 5	11 0	16 8	25 9	33 0	500	650	790	880	1020	1150
S	2 0	11 3	17 1	21 5	28 0	33 0	50	60	140	160	270	380
B	0 2	2 6	6 2	11 0	21 3	30 6	30	70	110	150	300	490
T	0 3	4 6	10 6	15 0	21 0	25 6	30	70	110	150	230	300
N	4 7	9 8	17 3	26 0	37 4	45 1	490	790	870	950	1090	1210
H	0	0 6	2 3	4 9	11 0	17 9	30	60	130	740	340	460
L	0 1	1 7	4 4	7 7	12 5	15 9	30	80	100	130	200	260
A	1 5	6 8	13 8	24 1	32 8	40 0	70	230	450	690	800	920
J	1 6	8 5	14 8	19 2	26 2	32 7	40	90	150	220	380	540
R	0 3	3 0	6 5	11 0	21 5	32 8	40	120	150	190	260	330
Mean	1 1	5 3	10 4	15 7	23 8	30 7	131	222	300	426	489	604
Tetracycline Hydrochloride												
E	3 5	11 9	19 5	25 3	34 2	42 9	370	640	730	830	970	1140
S	1 9	10 5	22 0	29 5	41 0	50 5	190	270	340	400	500	620
B	1 8	6 5	12 5	17 5	26 3	34 0	30	80	110	140	200	310
T	2 0	9 5	16 7	22 8	32 9	42 0	100	170	220	270	350	430
N	5 5	20 8	29 5	36 0	46 1	54 4	230	390	420	470	350	660
H	3 1	13 0	22 0	28 0	38 4	46 9	30	90	190	280	360	450
Mean	3 0	12 0	20 4	26 6	36 5	45.1	158	273	335	398	455	602
Tetracycline												
E	0 3	0 5	0 7	0 8	1 0	1 2	280	430	580	620	690	770
S	0.1	1 3	2 9	4 0	5 4	6 0	200	330	460	590	680	840
B	0.4	2 8	6 2	9.5	14 9	19 5	50	90	130	180	310	450
T	0	1 3	3 0	4.2	5 9	6 4	40	70	320	490	640	800
N	0	1 9	5 0	7 5	11 0	12 8	80	140	190	250	350	450
H	0 3	1 1	1 8	2 3	3 2	4 0	40	100	340	590	780	970
Mean	0 2	1.5	3.3	4.7	6 9	8 3	115	193	336	453	575	713

[a] All doses on the basis of 200 ± 10 mg tetracycline hydrochloride equivalent
[b] Bracketed numbers are subject's age in years followed by his weight in Kg

TABLE III.—EXCRETION DATA FROM SINGLE 200-MG. DOSES OF TETRACYCLINE IN THE FORM OF SMALL PARTICLES[a]

Cumulative mg. of Tetracycline and ml. Urine Excreted to Various Times in Hours

	mg. as Hydrochloride						Urine Volume, ml.					
Subject	1.0	2.0	3.0	4.0	6.0	8.0	1.0	2.0	3.0	4.0	6.0	8.0
	Tetracycline Hydrochloride											
E	2.5	10.4	17.4	23.0	31.9	39.4	250	570	670	790	940	1090
S	2.2	8.8	15.0	20.2	28.8	36.3	270	510	590	790	950	1100
B	1.1	6.0	13.0	21.5	32.2	39.7	30	50	70	120	220	330
T	2.2	7.2	14.2	22.5	34.8	44.3	50	80	130	180	260	350
N	0	3.3	10.4	21.0	38.3	52.0	70	150	190	230	300	370
Mean	1.6	7.1	14.0	21.6	33.2	42.3	134	272	330	422	534	648
	Tetracycline Sodium Hexametaphosphate Complex											
E	2.0	8.7	17.9	24.0	33.0	40.0	270	530	590	650	760	870
S	3.0	10.0	20.2	29.5	37.9	42.5	70	150	260	380	630	920
B	3.2	8.5	15.0	22.8	35.2	45.4	50	100	150	180	250	330
T	2.2	11.2	20.5	26.0	33.4	42.0	40	80	200	250	320	390
N	0	8.0	20.2	29.4	42.2	52.6	220	550	610	690	820	920
Mean	2.1	9.3	18.8	26.3	36.3	44.5	130	282	362	430	556	686
	Tetracycline											
E	2.5	9.3	16.2	21.2	28.9	34.9	320	640	770	900	1090	1180
S	2.9	8.8	17.1	27.2	46.0	59.6	170	600	790	970	1010	1220
B	2.1	6.3	11.8	17.2	25.5	32.0	70	110	130	160	240	320
T	1.0	7.2	17.9	27.0	39.4	49.0	50	100	150	200	270	330
N	1.0	5.0	11.7	20.5	36.5	48.2	600	710	780	840	910	960
Mean	1.9	7.3	14.9	22.6	35.3	44.7	242	432	524	614	704	802

[a] All doses on the basis of 200 ± 1 mg. tetracycline hydrochloride equivalent. Average diameter of particles, 100 μ.

It is not possible, in general, to obtain instantaneous excretion rates, hence average values must be used. In this paper, excretion rates are the average hourly values calculated from the slope of the mean cumulative amount *vs.* time excretion curve. For example, excretion rates at one hour represent the difference between the amounts excreted at one-half and one and one-half hours. When the interval for calculating mean rates was shortened to one-half hour, no substantial differences in values were found. The need for instantaneous rates becomes less important as the time of elimination and time for absorption increase. Tetracycline is eliminated rather slowly with a half-life in the blood stream of about eight hours (24).

Solution Rates in Vitro.—Table IV summarizes the values found with the materials and mediums used in tests. The average value shown was calculated by weighting the mean value in simulated intestinal fluids by a factor of 5. It was thought that a value weighted in this manner should more correctly represent the *in vivo* exposure of drugs to dissolution medium than a simple average of the *in vitro* rates.

TABLE IV.—*In Vitro* SOLUTION RATES[a]

	Simulated Mediums			
Material	Gastric	Neutral Intestinal	Alkaline Intestinal	Mean[b]
Tetracycline HCl	4.1	7.8	38	20
Tetracycline sodium hexametaphosphate complex	6.1	1.7	26	12
Tetracycline PSP	0.12	0.09	3.0	1.3
Tetracycline	2.6	< .001	<0.001	0.4

[a] mg./min. cm.²
[b] See text.

Correlation Between Excretion Rate and in Vitro Solution Rate in Pellet Tests.—The cumulative amounts excreted and excretion rates found when the pelleted preparations were taken are shown in Figs. 1 and 2. An examination shows a marked difference among the preparations tested both in

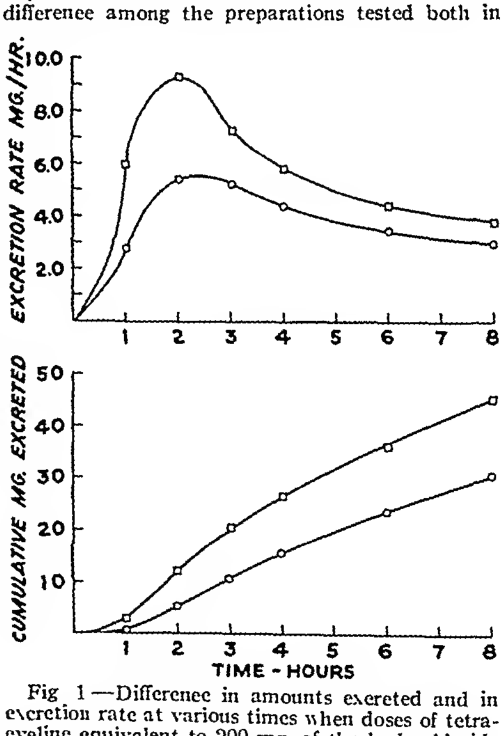

Fig. 1.—Difference in amounts excreted and in excretion rate at various times when doses of tetracycline equivalent to 200 mg. of the hydrochloride were taken in the form of pellets as the sodium hexametaphosphate complex (open circles) and as the hydrochloride itself (open squares).

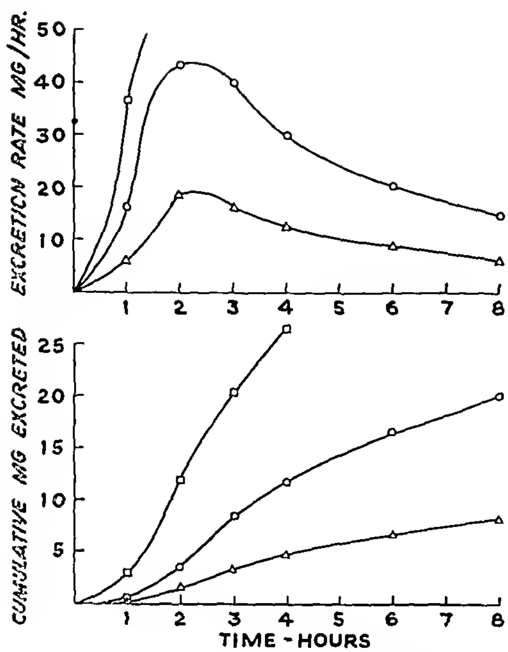

Fig 2—Difference in amounts excreted and in excretion rate at various times when doses of tetracycline equivalent to 200 mg of the hydrochloride were taken in the form of pellets as the PSP salt (open circles) and as tetracycline (open triangles) Curve for the hydrochloride from Fig 1 included for reference (open squares)

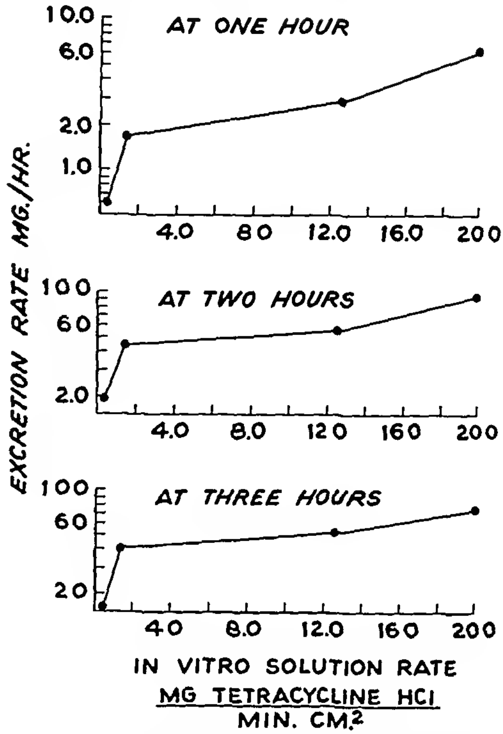

Fig 3—Correlation between excretion rate at one, two, and three hours and *in vitro* solution rate when various tetracyclines were taken in the form of pellets Points, from left to right on all diagrams are, respectively, tetracycline, tetracycline PSP, tetracycline sodium hexametaphosphate complex, and tetracycline hydrochloride

total amounts excreted and excretion rates at any given time When the excretion rates at one, two, and three hours were plotted against the solution rates determined *in vitro*, a definite correlation was seen to exist between these quantities This is shown in Fig 3 This comparison is arbitrary, but it does indicate qualitatively the dependence between the quantities

The initial surface area presented to gastrointestinal fluids when these preparations were taken was about 2 8 sq cm Under these conditions, there can be no doubt that *in vivo* solution rate was rate limiting in absorption

In the curves shown on Figs 1 and 2, the data were obtained from ten subjects in the case of the sodium hexametaphosphate complex and PSP salts and from six subjects (included in the ten) in the other cases No substantial change in the curves resulted when the mean excreted amounts from the basic six subjects only were used This will be discussed further, later in this report

Excretion Rate from Small Particle Preparations.—An examination of Fig 4 shows that when tetracyclines were given in a form where intial surface was relatively large as compared to the pellets, the mean amounts excreted and mean excretion rates were almost the same The particles used to fill the capsules here had an average diameter of about 100 μ On the basis that all particles were spherical with this diameter and possessed a density of 1 4, the initial surface area for tetracycline hydrochloride contained in capsules was about 85 sq cm For tetracycline sodium hexametaphosphate complex the value of the same quantity was about 115 sq cm, since this material is less active than the hydrochloride salt There was a significant difference in amounts excreted at three hours between these two preparations as will be discussed later

Comparison of Excretion Rates from Tetracycline Salts in Pellets and as Small Particles.—The combined effects of intrinsic solution rate and initial surface area are well illustrated in Figs 5, 6, and 7 With tetracycline, which possessed the slowest intrinsic solution rate of all the materials tested, the effect of increased surface area was extremely pronounced Since this material dissolved quite rapidly in simulated gastric fluid, it is likely that in most cases the drug dissolved in the stomach when the capsules were taken The necessity for large initial surface areas became less important with increase in intrinsic solution rate of the material used as is illustrated in the curves of Figs 6 and 7, respectively, for the sodium hexametaphosphate complex and hydrochloride salts The differences between excretion rates from pellets and capsules decreased as intrinsic solution increased In fact, it appeared that the initial surface area needed with the hydrochloride salt was no greater than that available in the pellets of this material, since excretion rate was actually greater with pellets than with 100 μ particles It was apparent from these results that *in vivo* solution rate was not overwhelmingly rate limiting in absorption when the hydrochloride salt was used

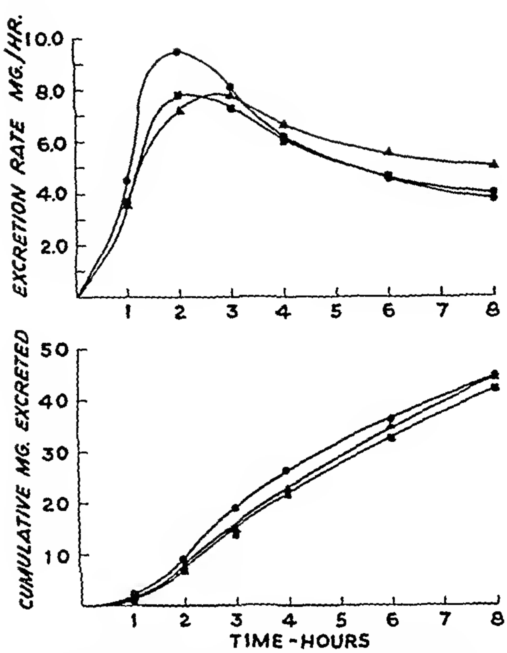

Fig. 4.—Nature of cumulative amount excreted and excretion rate curves when two tetracycline salts and tetracycline were taken in capsules containing particles with 100 μ average diameter. Solid squares—tetracycline hydrochloride, solid circles—tetracycline sodium hexametaphosphate complex, and solid triangles—tetracycline. All doses equivalent to 200 mg. of tetracycline hydrochloride.

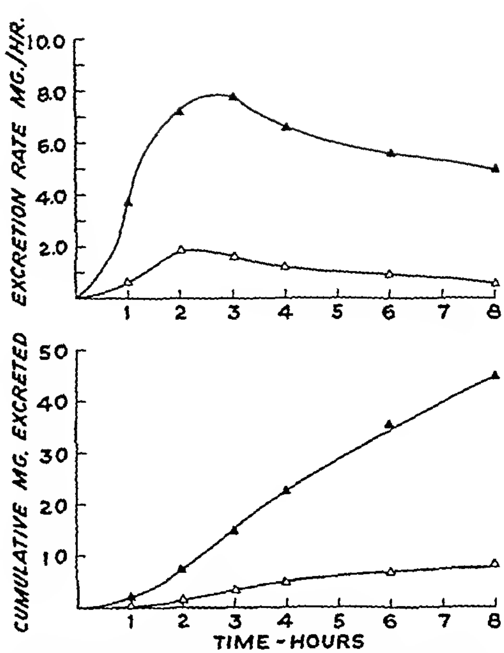

Fig. 5.—Difference in cumulative amount excreted and excretion rate curves when tetracycline was taken as pellets and as particles with average diameter of 100 μ. Dose: 200 mg. tetracycline hydrochloride equivalent. Solid triangles—tetracycline as 100 μ particles and open triangles—as pellets.

Significance of Differences in Results.—An examination of the individual entries in Tables II and III shows that relatively large variations occurred even though the means of entries indicated definite trends as has been discussed in preceding sections. The means of amounts excreted to one, two, and three hours were tested for significant differences by the t test and the results are summarized in Table V.

Since there was a significant difference between mean amounts excreted when pellets of the hydrochloride and sodium hexametaphosphate complex were compared, the results were significant between tetracycline hydrochloride and all the other materials used in pellets. The differences between the hydrochloride and sodium hexametaphosphate existed also in the case where the comparison was made with the basic six crossover subjects.

The differences between mean amounts excreted of tetracycline sodium hexametaphosphate complex and the PSP salts were not significant in spite of the fact that the excretion rates of these two materials were of the correct order in comparison to their *in vitro* solution rates. An examination of Fig. 3 shows, however, that the excretion rate of tetracycline PSP was much more rapid than its *in vitro* solution rate should allow when considered with the other materials. It was likely that the *in vivo* solution rate differences between these two substances were much less than that the differences indicated by the results of *in vitro* measuremets.

It might be thought that an explanation for the significant differences in

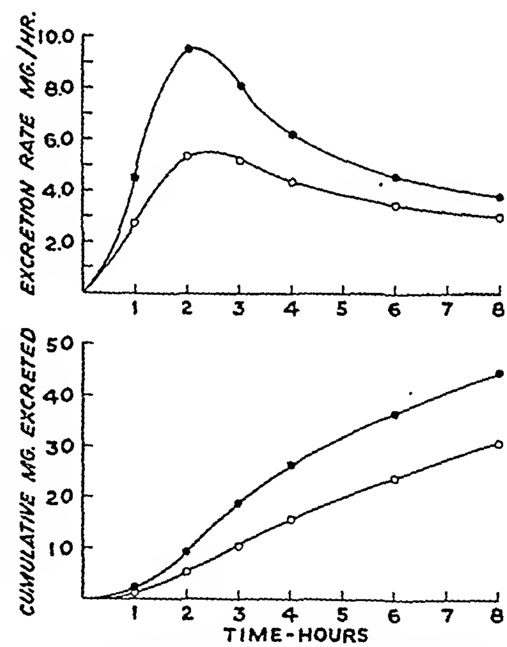

Fig. 6.—Difference in cumulative amount excreted and excretion rate curves when tetracycline sodium hexametaphosphate complex was taken as pellets and as particles with average diameter of 100 μ. Dose: 200 mg. tetracycline hydrochloride equivalent. Solid circles—100 μ particles and open

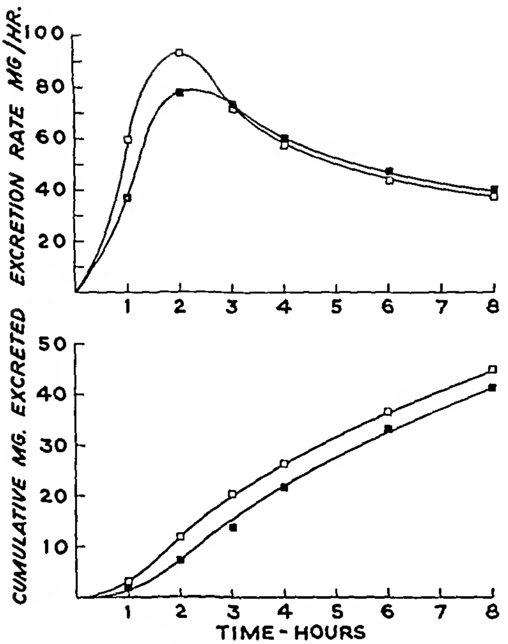

Fig 7 —Illustrating a case, by cumulative amount excreted and excretion rate curves, where absorption of tetracycline was not rate-limited by *in vivo* solution rate Dose in each case was 200 mg tetracycline hydrochloride Solid squares—as 100 μ particles and open squares—as pellets

three hours from small particles of the sodium hexametaphosphate complex and the hydrochloride was in the differences in the products of *in vitro* solution rate in simulated gastric fluid and surface area between the two materials However, other results indicated that *in vivo* solution rate was not rate-limiting in absorption when capsules containing small particles were taken Furthermore, pellets of hydrochloride gave mean rates greater than capsules of the sodium hexametaphosphate complex

The last entry in Table V indicates the significance of the differences in mean amounts found when comparing absorption of tetracycline HCl from pellets and capsules It seems reasonable to assume that the occurrence was due to chance

Dose Dispersion.—The fact that tests with capsules containing relatively fine material gave nearly identical results when the hydrochloride, sodium hexametaphosphate complex, and free base were compared, does not necessarily mean that capsules containing powdered materials would always give the same results In these tests all surface could be considered to have been made available for contact with gastrointestinal fluids due to the bicarbonate content Addition of materials that would tend to prevent dispersion of capsule contents could be logically expected to hinder *in vivo* solution and, hence, absorption

Sodium bicarbonate or effervescent mixtures based on sodium bicarbonate have been employed to increase the rate of absorption of sulfathiazole and acetylsalicylic acid (26) It was shown that when about 2 0 Gm of sodium bicarbonate were given with these drugs, the rate of build-up of blood concentration with time was much more rapid than

TABLE V —SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS OF AMOUNTS EXCRETED FROM SEVERAL PREPARATIONS (t TEST)

	1 0 Hour	2 0 Hours	3 0 Hours
	Pellets of Tetracycline Hydrochloride and Sodium Hexametaphosphate Complex (14 Degrees of Freedom, t = 2 15[a])		
t, calculated	2 48	3 31	3 56
	Pellets of Tetracycline Hydrochloride and Sodium Hexametaphosphate Complex (10 Degrees of Freedom, t = 2 23[a])		
t, calculated	1 78	2 47	2 88
	Pellets of Tetracycline Sodium Hexametaphosphate Complex and PSP (18 Degrees of Freedom, t = 2 0[a])		
t, calculated	1 27	1 65	1 39
	Pellets of Tetracycline PSP and Tetracycline (14 Degrees of Freedom, t = 2 15[a])		
t, calculated	1 56	3 66	2 64
	Capsules of Tetracycline Sodium Hexametaphosphate Complex and Hydrochloride (8 Degrees of Freedom, t = 2 31[a])		
t, calculated	0 65	1 58	3 07
	Pellets and Capsules of Tetracycline Hydrochloride (9 Degrees of Freedom, t = 2 26[a])		
t, calculated	1 95	2 00	2 34

[a] Value to be exceeded before difference between means can be considered significant (95% confidence)

when it was omitted It was explained that bicarbonate hastened stomach emptying and thereby transferred the drug to the intestines where it would be better absorbed Although stomach emptying time is a factor in tetracycline absorption as a consequence of the pH-partition hypothesis (14–16), the dose used in the present work was tenfold less than the dose previously employed for increasing emptying rate (26)

The effervescence produced by contacting sodium bicarbonate and acidic gastric fluid could, on the basis of the solution rate concept advanced here, be expected to "enhance" absorption in those cases where absorption was rate-limited by surface and intrinsic solution rate This view is reasonable when the marked effect of agitation on solution rate is considered In fact, increased agitation seems a reasonable explanation for the sulfathiazole and acetylsalicylic acid work (26)

Solution Rate.—The differences in *in vitro* solution rate found here were due to the influence of the dissolving material on its diffusion layer This influence has been discussed in previous publications concerning weak acids (10, 11) The situation is analogous with a material such as tetracycline even though its basic function was used in salt formation

Tolerance to Tetracycline by Subjects.—All subjects tolerated the tetracycline dosages well In two or three instances, slight stomach upset was noticed which could be attributed to either sodium bicarbonate or tetracycline One subject developed a moderately severe case of urticaria after dosage

with pellets of tetracycline hydrochloride. However, continuation in the testing program by this subject after a two-week lapse of time was without further reaction to drug.

It has been reported that tetracycline taken in tablets results in more frequent side effects such as nausea, generalized itching, diarrhea, and pruritus ani than tetracycline taken in capsules (27). The present work may offer an explanation for these occurrences Generally speaking, capsules may be considered as "high availability" dosage forms when compared to tablets Degree of difference depends, of course, on binders, excipients, etc, used Those side effects arising from disturbances in the gastro-intestinal tract could be due to retention of undissolved drug in tablets in the intestinal tract for long periods of time. In the few tests that were made here where excretion was followed to twenty-four to twenty-eight hours, substantial amounts of pelleted preparations sometimes remained unexcreted when compared to amounts of tetracycline hydrochloride excreted in the same period This was the case in particular with tetracycline pellets

SUMMARY

When tetracycline absorption was studied by means of excretion rate determinations, it was found that intrinsic solution rate was rate-determining in absorption providing initial surface area was restricted in the dosage form. With increased surface area of drug in the dosage form and with provision made to make this surface available to dissolution medium *in vivo*, it appeared that overall solution rate was not rate-limiting in absorption of this drug and its salts. It was also shown that excretion rate studies were capable of supplying comparative information on the blood levels of drug.

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Studies on Glutamic Acid Decarboxylase*

By R. P. SMITH,† G. E. CWALINA, and E. RAMSTAD

The presence of glutamic acid decarboxylase activity was demonstrated in extracts of *Papaver somniferum* and *Ricinus communis*. The product of this reaction, 4-aminobutyric acid, was found to be present in *Datura stramonium* and *Delphinium ajacis*. It was identified as the only product of glutamic acid metabolism in squash preparations and was not metabolized further.

Although considerable interest was aroused by the announcement of the discovery of glutamic acid decarboxylase in brain tissue of rats and mice (1-6), and in spite of the fact that this enzyme is by far the most predominant amino acid decarboxylase found in biological systems (7-11), neither its function nor the metabolic fate of the reaction product, 4-aminobutyric acid, are known at this time.

Davison (12) has offered some evidence that different enzymes are responsible for the decarboxylation of glutamic acid by rat brain and rat liver preparations.

Rogers (13) has shown that of 35 common amino acids and acids concerned with respiration only L-(+)-glutamic acid was strongly decarboxylated by acorn squash preparations. The amino group appears to be necessary for decarboxylation since 2-ketoglutaric acid is not decarboxylated under the conditions used.

Naylor and Tolbert (14) have studied glutamic acid decarboxylase in green and etiolated barley leaves. They found the rate of glutamic acid

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metabolism was faster in the light than in the dark Through the use of isotope tracer technique they also established that 80 per cent of the glutamic acid metabolized under anaerobic conditions was decarboxylated to 4-aminobutyric acid, and that 4-aminobutyric acid was the only product *in vivo* from the action of glutamic acid decarboxylase on glutamic acid

EXPERIMENTAL

Manometric Approach to Glutamic Acid Decarboxylase Activity.—Homogenates were prepared by mixing the fresh plant material in a Waring Blendor at 5° in the presence of an appropriate phosphate buffer and followed by filtration The Warburg Constant Volume Respirometer was used to measure carbon dioxide evolution directly in an atmosphere of purified nitrogen according to a standard procedure (15) The bath temperature was 37 5° and the shaking rate was 116 per minute with a stroke of 3 cm

Using the method of Rogers (13), it was found that 100 Gm of acorn squash pulp extracted with 100 cc of phosphate buffer of pH 5 8 gave favorable glutamic acid decarboxylase activity when a 3 0 cc aliquot was used with 1 0 cc of L-(+) glutamic acid containing 33 micromoles per cc

Applying the above conditions to whole two-month-old *P somniferum* seedlings, a significant[1] amount of carbon dioxide evolution was observed in a set of eight experimental determinations over the controls Glutamic acid decarboxylase activity was then observed in *R communis* preparations of two-month-old seedlings under the same experimental conditions although it was necessary to increase the sample size to a set of 12 determinations to obtain the same level of significance The results of these experiments are summarized in Fig 1 *P somniferum* was then tested under the above conditions for decarboxylase activity against substrates of DL-tryptophan, DL phenylalanine, DL-tyrosine, and DL-dopa No significant carbon dioxide evolution was noted

Manometric Approach to Glutamate and 4-Aminobutyrate Metabolism.—Squash homogenates as previously described were tested for 2-ketoglutaric acid decarboxylase activity both with and without the presence of 66 micromoles of methylene blue in each reaction vessel to act as a hydrogen acceptor No decarboxylase activity could be established in either case

Attempts to demonstrate oxygen uptake by squash homogenates and a 4 aminobutyrate substrate were unsuccessful It was not possible to demonstrate the formation of succinate or succinyl semialdehyde by chromatography of these reaction mixtures

It was demonstrated by standard techniques (15) that the anaerobic decarboxylation of glutamic acid by squash homogenates exceeds the aerobic decarboxylation reaction The system employed consisted of 1 8 cc of the standard squash homogenate, 0 5 cc of the standard substrate solution in an atmosphere of air for the aerobic set and in purified nitrogen for the anaerobic set A filter paper fan and 0 2 cc of 10% KOH were used in one flask of the aerobic set to take up the carbon dioxide evolved This 0 2 cc volume was equalled in all other flasks by additional buffer Carbon dioxide evolution was observed at two different pH values, the results are summarized in Fig 2

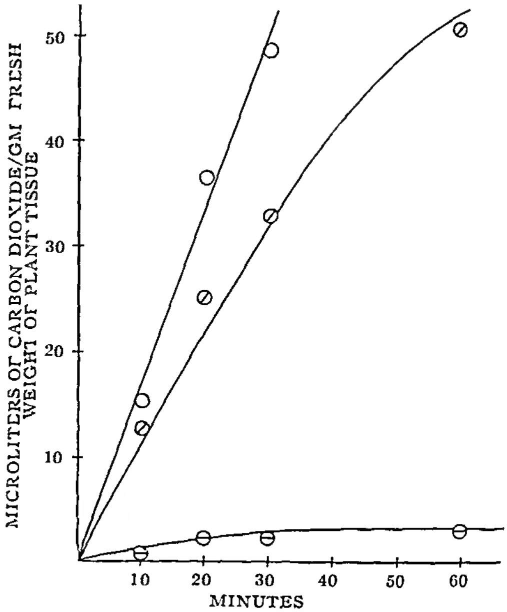

Fig 1 —Cumulative carbon dioxide evolution from equivalent preparations of three different plant materials using an L-(+) glutamate substrate O Acorn squash, ⦶ *P somniferum*, ⊖ *R communis*

Chromatographic Studies.—Paper partition chromatography was adopted in this work as a check on the manometric results A two dimensional ascending system of *n*-butanol/acetic acid/water (4 4 1) followed by phenol saturated with water was used The chromatograms consisted of sheets of Whatman #1 filter paper, 20 cm square, and the material was spotted in one corner 5 cm from each edge The chromatograms were stapled into cylinders and placed upright on the bottoms of commercial cylindrical chromatography chambers

For more positive identification unknowns co spotted satisfactorily in the above systems were run in *tert*-butanol/methyl ethyl ketone/water (4 4 2) followed by *tert*-butanol/methanol/water (4 5 1) in the second direction All of the chromatograms were developed by spraying with 0 1% ninhydrin in chloroform, or, if acids of interest were suspected to be present, the ninhydrin spraying was preceded by 0 04% bromocresol green dissolved in 95% ethanol

In the above described fashion, 4-aminobutyric acid was identified as the main reaction product with glutamic acid in acorn squash and *P somniferum* homogenates chromatographed directly after runs in the Warburg apparatus Free 4-aminobutyric acid was also found to be present in equivalent preparations of *R communis, Datura stramonium,* and *Delphinium ajacis*

Radioactive Tracer Studies.—2-C[14]-labeled

[1] Criteria established at the 5% confidence level using the *t* distribution of Dixon and Massey, "Introduction to Statistical Analysis," McGraw Hill Book Co, Inc, New York, 1951

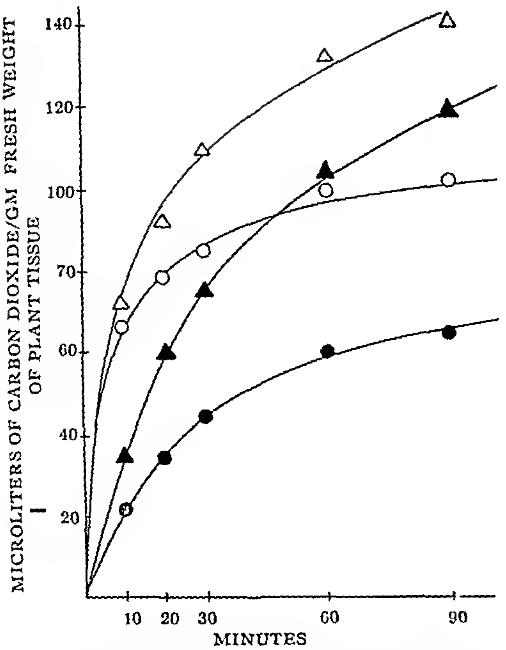

Fig 2 —A comparison of the aerobic and anaerobic cumulative carbon dioxide evolution from acorn squash preparations at pH 5.8 and 7.4 using a L-(+)-glutamate substate ○ Anaerobic, pH 7 4; ● Aerobic, pH 7.4; △ Anaerobic, pH 5 8, ▲ Aerobic, pH 5.8

glutamic acid was obtained having a specific activity of 2.86 μ c. per mg. Two milligrams of this material was dissolved in 1 0 cc. of a buffer of pH 5 8 and enough carrier glutamate was added to bring the concentration up to 33 micromoles per cc. This volume of substrate was run with 3 0 cc. of freshly prepared squash homogenate in a nitrogen atmosphere at 37.5°. After one hour, the reaction mixture was poured into an equal volume of boiling alcohol and filtered Appropriate aliquots were spotted and chromatographed in the previously described manner.

Autoradiograms were prepared from the developed chromatograms and it was established that 4-aminobutyric acid was the sole reaction product from this preparation and that it was not metabolized further.

DISCUSSION

The role of 4-aminobutyric acid in metabolism and its subsequent fate has thus far successfully eluded investigators All indications seem to point toward its being a waste product in the system employed. The Warburg studies on aerobic and anaerobic decarboxylation supports the findings of Tolbert and Naylor (14) who observed the phenomena of increased decarboxylation under anaerobic conditions in barley leaves by radioactive tracer technique. Their suggestion that the further metabolism of 4-aminobutyric acid is inhibited by anaerobiosis is given an unfavorable light by the evidence here presented that 4-aminobutyrate is not metabolized further under either aerobic or anaerobic conditions in the system employed.

We were not able to observe the decarboxylation reaction in intact tissue as repeated imbibition experiments with glutamic acid gave no results

We wish to draw attention to the biochemical oddity demonstrated by autoradiography in that glutamic acid has a sole metabolic product in the system employed In fact, this method was used for preparing radiochemically pure samples of 4-aminobutyric acid Much criticism could be offered to the choice of a resting fruit for metabolism studies, but the choice was made from a viewpoint of economy, convenience, and degree of enzymatic activity It may well be that such reactions do not occur in normal metabolism, but, if so, this work at least shows what metabolic abnormalities may occur in resting organs.

CONCLUSIONS

1. Enzyme preparation extracts from *Papaver somniferum*, *Datura stramonium*, and *Ricinus communis* were screened for amino acid decarboxylase activity under a variety of conditions and with several substrates

2. Glutamic acid decarboxylase activity was observed manometrically in homogenates of whole seedlings of *P. somniferum* and *R. communis*.

3. 4-Aminobutyric acid was observed to be the main reaction product from glutamic acid addition to homogenates of *P somniferum* The presence of free 4-aminobutyric acid was established in extracts of *R communis*, *D. stramonium*, and *Delphinium ajacis*.

4 The anaerobic decarboxylation of glutamic acid by acorn squash pulp homogenates was found to exceed the reaction under aerobic conditions at pH 7.4 and 5.8.

5. 4-Aminobutyric acid was identified as the only product of glutamic acid metabolism in squash preparations.

6. 4-Aminobutyric acid was shown not to be metabolized by squash preparations.

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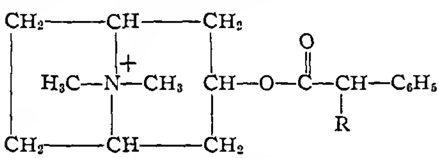
The Kinetics of the Hydrolysis of Homatropine Methylbromide and Atropine Methylbromide*

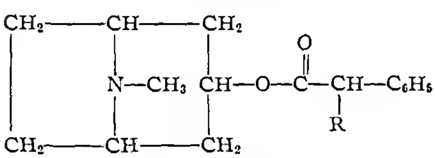
By J. L. PATEL† and A. P. LEMBERGER

A study has been made on the chemical kinetics of the hydroxyl ion catalyzed hydrolysis of homatropine methylbromide and atropine methylbromide. The temperature dependency of the hydrolytic reactions has been determined and Arrhenius equation constants are evaluated. Mandelyl tropine esters having an α-hydroxyl group are found to hydrolyze at a rate approximately five times that of the corresponding tropyl tropine esters. The positive charge in quaternary tropine esters has been shown to effect a thirty-five-fold increase in the rate of tropine ester hydrolysis.

A REPORT on the kinetics of the hydrolysis of homatropine has already been presented (1) as a part of an investigation to evaluate quantitatively the contribution of polar and steric factors and the positive ionic charge on the rate of tropine ester hydrolysis. This publication reports the kinetics of hydrolysis of homatropine methylbromide (I) and atropine methylbromide (II) and correlates the data with that previously obtained for homatropine (III) and atropine (IV) (2).



I. R = OH
II. R = CH_2OH



III. R = OH
IV. R = CH_2OH

THEORETICAL CONSIDERATION

Unlike homatropine, the mechanism of hydrolysis of homatropine methylbromide and atropine methylbromide remains the same throughout the entire pH range. Specific rate constants were therefore determined only in moderately alkaline solutions. Half-life periods for these quaternary salts can be predicted from the specific rate constants for any given hydroxyl ion concentration. The pH dependency studies were therefore not carried out. Determinations were made at temperatures ranging from 10 to 25.8°, and Arrhenius equation constants were evaluated.

Determination of homatropine methylbromide and atropine methylbromide in the presence of their degradation products is a difficult task. High water solubility of these esters limits their separation from hydrolysis products and the method used by Zvirblis, *et al.* (2), for atropine does not work. Bandelin (3) determined homatropine methylbromide by precipitating a reineckate complex, which was then dissolved in acetone and measured colorimetrically. Robinson (4) found that in the reineckate procedure, tropanol methylbromide (a hydrolysis product of homatropine methylbromide and atropine methyl bromide) yielded the same amount of color when complexed with ammonium reineckate. Robinson used Valser's reagent to assay homatropine methylbromide and reported that the absorbances of aqueous suspensions of methyl tropanol iodo-mercurate and methyl homatropine iodo-mercurate complexes differed sufficiently to allow measurement of the degree of hydrolysis of homatropine methylbromide. The typical Beer's law plot of the absorbance of the methyl homatropine iodo-mercurate complex in solution *versus* concentration as illustrated by him is not linear and the method was found to be unsatisfactory for the present investigation.

Another way of determining the bimolecular rate constants of alkaline hydrolysis of ester is to transfer aliquots from the reaction mixture into excess acid at suitable time intervals and back titrate with alkali to the appropriate end point. Garrett (5) has used the titration method for the study of the kinetics of the hydrolysis of scopolamine derivatives. For some scopolamine derivatives, rates were very high and the reaction was complete in three to four minutes. An attempt to study the hydrolysis of homatropine methylbromide by the above method showed that the ester reacted almost completely in 0.01 N alkali in about two minutes at room temperature. More dilute alkali was tried to reduce the rate of reaction and to facilitate the periodical removal of the aliquots from the reaction mixture. In the dilute alkali, however, the indicator end point was found to be irreproducible. Using photometric titration for the determination of substituted phenols, Goddu and Hume (6) have shown that photometric titration has an advantage over potentiometric methods when determinations are made in highly dilute solutions and with very weak acids. This led to the use of photometry, the change in absorbance of an indicator as a function of hydroxyl ion concentration, to follow the progress of the reaction. To serve this purpose, the indicator should show color changes in the selected alkali concentration, should be reasonably stable in alkali, and its acid form should not absorb

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† Present address: Alembic Chemical Works, Baroda, India.

appreciably at the absorption wavelength of the base form. Alizarin yellow R (sodium-*p*-nitrobenzene-azo-salicylate) was tried and found to fulfill the said requirements and, hence, was chosen as an indicator for the present investigation. The possibility that the ester formed a complex with the indicator seems very unlikely since the plots conformed to bimolecular kinetics.

For each indicator there is a characteristic zone of hydrogen ion concentration, on the acid side of which the indicator is completely transferred into its acid form and on the alkaline side into its base form. Within this range, there will be different proportions of the acid and base forms. If the indicator is a weak acid, its ionization can be represented by

$$HIn \rightleftarrows H^+ + In^-$$

and the corresponding equilibrium constant is

$$K_a = \frac{(H^+)(In^-)}{(HIn)} \qquad \text{(Eq. 1)}$$

or

$$(OH^-) = K_b \frac{(In^-)}{(HIn)} \qquad \text{(Eq. 2)}$$

where K_a and K_b are the ionization constants of the acid and base form of the indicator, respectively.

Indicator base-acid ratios were calculated from its absorbance data by means of the equation

$$\frac{(In^-)}{(HIn)} = \frac{A - Aa}{Ab - A} \qquad \text{(Eq. 3)}$$

where A = absorbance during reaction; Aa = absorbance of pure acid form of indicator; and Ab = absorbance of pure base form of indicator. This approach is similar to the type III plots described by Rehm and Higuchi (7).

EXPERIMENTAL

Reagents.—Homatropine methylbromide U. S. P.; atropine methylbromide, K and K Laboratories, Inc., recrystallized from ethanol, m. p. 220–221°; approximately 0.01% alizarin yellow R in water containing 5% ethanol.

Alkaline Hydrolysis of Homatropine Methylbromide and Atropine Methylbromide.—Homatropine methylbromide was studied in solution 0.0066 *M* and 0.005 *M* in ester and 0.0066 *N* in barium hydroxide. Atropine methylbromide was studied in solution 0.0066 *M* in ester and 0.0066 *N* in barium hydroxide. Five milliliters of indicator solution and 25 ml. of barium hydroxide solution were placed in a thermostatically controlled bath and allowed to reach temperature equilibrium. A weighed amount of ester was then added and rapidly mixed with the solution. The reaction mixture was periodically transferred into the cell of a Cary model 11 recording spectrophotometer and the absorbance at wavelength 493 mμ was recorded. For room temperature studies, the reaction was allowed to take place in the spectrophotometer cell after the first transfer. Studies were made at 10, 15, 20, and 25.8°, to allow calculation of the Arrhenius constants.

Absorbance of the solution containing 5 ml. of indicator solution and 25 ml. of barium hydroxide solutions of varying strengths served as a blank to calculate hydroxyl ion concentration during the course of reaction. All the determinations were made in an atmosphere of nitrogen.

RESULTS AND DISCUSSION

The kinetics of the alkaline hydrolysis of the ester groups in homatropine methylbromide and atropine methylbromide were determined by estimation of the slopes appropriate to the bimolecular rate expressions

$$\frac{1}{a - x} = kt + \text{constant} \qquad \text{(Eq. 4)}$$

$$\log \frac{b}{a}\,\frac{a - x}{b - x} = \frac{kt\,(a - b)}{2.303} \qquad \text{(Eq. 5)}$$

Thus $\frac{1}{a-x}$ was plotted against the time when $a = b$ and $\log \frac{b}{a}\frac{a-x}{b-x}$ was plotted against time when $a \neq b$ where a and b are the initial concentrations and $(a - x)$ and $(b - x)$ are the concentrations at time intervals for the alkali and ester, respectively. Hydroxyl ion concentration was calculated from the absorbance data and ionization constant of the indicator using Eqs. 2 and 3. Calculation of the ester concentration was based on the postulated 1:1 reaction of ester with hydroxide ion. A typical blank plot showing the validity of Eq. 2 is shown in Fig. 1. Experimentally determined ionization constants of the base form of the indicator are given in Table I.

As illustrated in Figs. 2 and 3, the observed rate of hydrolysis of homatropine methylbromide and atropine methylbromide is second order. The bimolecular rate constants (k) in liter mole^{-1}second^{-1} are given in Table II.

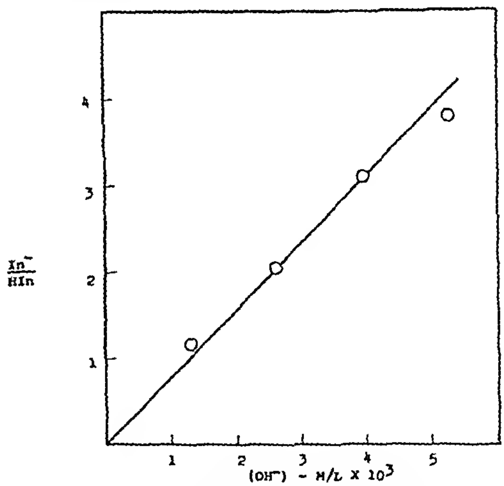

Fig. 1.—Blank plot relating the indicator absorbance with the hydroxyl ion concentration at 25.8°.

TABLE I.—IONIZATION CONSTANTS OF ALIZARIN YELLOW R, BASE FORM

Temp. °C.	$K_b \times 10^3$
10.0	1.10
15.0	1.15
20.0	1.22
25.8	1.31

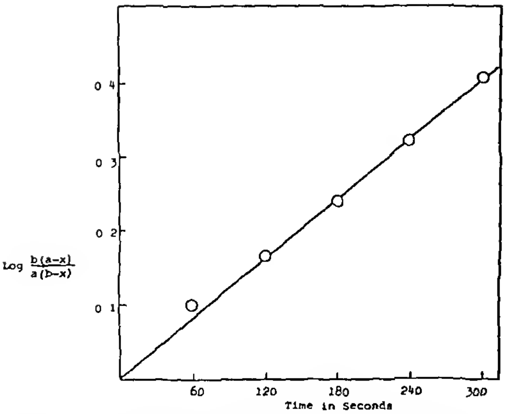

Fig 2 —Typical bimolecular rate plot for the alkaline hydrolysis of homatropine methylbromide at 25 8°

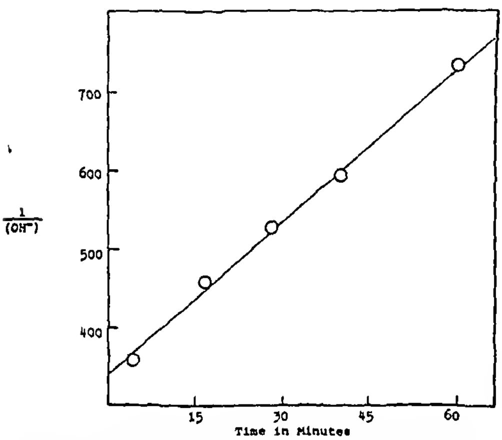

Fig 3 —Typical bimolecular rate plot for the alkaline hydrolysis of atropine methylbromide at 10°

TABLE II [a]—BIMOLECULAR RATE CONSTANTS FOR ALKALINE HYDROLYSIS OF HOMATROPINE METHYLBROMIDE AND ATROPINE METHYLBROMIDE

	k in liter mole^{-1} sec^{-1}	
Temp °C	Homatropine Methylbromide	Atropine Methylbromide
10 0	0 53	0 11
15 0	0 78	0 17
20 0	1 11	0 26
25 8	1 74	0 37

[a] Calculated bimolecular rate constants in liter mole^{-1} sec^{-1} for homatropine and protonated homatropine at 25 8° are 0 039 and 1 54 respectively (1)

Temperature Dependence—Rate constants for homatropine methylbromide and atropine methylbromide were determined at four different temperatures Bimolecular rate constants are plotted against the reciprocal of the absolute temperature in Figs 4 and 5 The activation energy was calculated by setting the slope equal to $-E/2\ 303R$ and the frequency factor (s) in the Arrhenius equation was calculated using $k = se^{-E/RT}$ The constants of the Arrhenius equation are given in Table III

A significant difference in the specific rate constants is observed when the values for various tropine

TABLE III —TABULATION OF CONSTANTS OF THE ARRHENIUS EQUATION

Compound	E Kcal /Mole	Log S^a
Homatropine methylbromide	13 0	9 74
Atropine methylbromide	14 0	9 82

[a] Log $k = -E/2\ 303\ RT + \log S$ where k is in liter mole^{-1} sec^{-1}

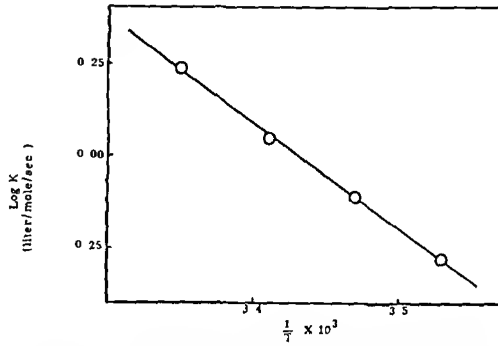

Fig 4 —Arrhenius plot of the bimolecular rate constants for the alkaline hydrolysis of homatropine methylbromide

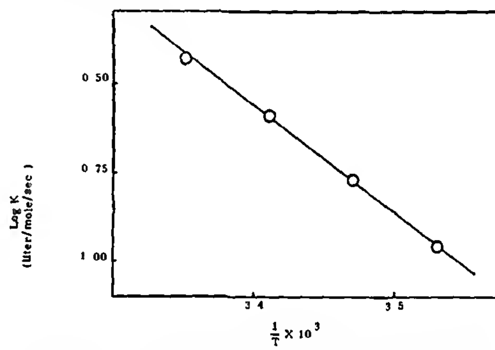

Fig 5 —Arrhenius plot of the bimolecular rate constants for the alkaline hydrolysis of atropine methylbromide

esters are compared Ingold (8) has shown the retarding effect of alkyl substituents and negative ionic charge and the accelerating influence of electron attracting Cl, OH, $COCCH_3$, $COCH_3$, CH_2COCH_3, and CH_2OCH_3 groups on the alkaline hydrolysis of aliphatic carboxylic esters The difference in rates has been attributed by this author clearly to the polar effects, whatever the steric effects may be Garrett (5) in his studies on the kinetics of scopolamine derivatives has attributed the increased rates in positively charged quaternary salts to the field effects

In the case of tropine esters, it is found that mandelyl tropine esters, *viz* base and acid form of homatropine and homatropine methyl bromide, degrade at a rate approximately five times that of the corresponding tropyl tropine esters, *viz* the base and acid form of atropine and atropine methyl bromide The accelerating effect of the α-hydroxyl group in mandelyl tropine esters is in agreement with the scheme postulated by Waters (9) and Ingold and Ingold (10) to explain the alkaline hydrolysis of esters

Comparison of the rate constants of protonated homatropine, homatropine methylbromide, and atropine methylbromide with the corresponding free bases has shown that positive charge on nitrogen in quaternary salts enhances their rate very significantly. The 35-fold increase in the rates in these quaternary tropine esters may be attributed to the field effects resulting in increased concentration of hydroxide ion in the neighborhood of the ester molecule.

Thus, it is observed that the polar and/or steric effects have a great influence in determining the rate of alkaline hydrolysis of highly substituted esters such as tropine esters.

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The Effect of Adipic Acid and Methylamine on Alkaloid Biosynthesis in Certain Members of the *Solanaceae* I*

Datura stramonium Linné

By M. S. BROOKS, L. A. SCIUCHETTI,† and D. P. N. TSAO

Adipic acid administered by injection in the concentration of 0.5 per cent increased the total alkaloids (calculated as hyoscyamine) in the leaves (and flowering tops), stems, and roots by 14, 26, and 79 per cent, respectively. Methylamine in the same concentration increased the total alkaloid yield in the leaves and roots by 10 and 39 per cent while a 12 per cent decrease was observed in the total alkaloids of the stems. The plants treated with adipic acid and methylamine displayed an increase of alkaloid yields in the leaves and roots by 22 and 65 per cent while no change in alkaloid production in the stem was observed.

ROBINSON'S method (1) of synthesis of tropine has been applied to the synthesis of a number of tropinone-like compounds by replacing the succinic dialdehyde with various aldehydes such as adipic dialdehyde (2). Adipic acid was found in the juice of the sugar beet by Vavruch (3). Kameda and Toyoura (4) reported that adipic acid has been utilized by soil bacteria as a source of carbon in culture media. Dagley and Rodgers (5) have reported that intact cells of a certain vibrio grown at the expense of adipic acid gave high initial rates of oxidation of citrate. Since adipic acid and methylamine have been proposed as starting materials for the biosynthesis of tropine by Trautner (6), it was deemed desirable to study the influence of these two chemicals on alkaloid biosynthesis in *Solanaceous* plants, especially *Datura stramonium*.

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EXPERIMENTAL

Procedure.—Stramonium plants employed in this study were grown under carefully controlled greenhouse conditions.[1] Seeds were germinated in culture flats containing soil composed of two parts of compost and one part of peat moss on December 29, 1955. Sixty seedlings were transplanted into pots on June 26, 1956. On August 21, 1956, twenty-four plants of approximately the same height and in similar states of growth were selected for this study and divided into four groups. Each group consisted of six plants. One group was designated as control or untreated. The remaining three groups were treated with 0.5% solutions of adipic acid, methylamine HCl, and a combination of adipic acid and methylamine HCl. Each plant of the treated groups received 3–4 ml. of the respective solutions at a rate of about 2 ml. per hour by injection into the stem (7). Two injections were given to each plant of the treated group. The plants of the control group received no injection. The first injection was administered on August 21, 1956. A second injection was given on the following day. No observable toxic effects were noted in the treated plants

[1] The authors are indebted to Dr. W. R. Naumann and Mr. M. Bakke of Drug Plant Gardens and Laboratories, College of Pharmacy of the University of Washington, for their generous assistance during the growth of Datura plants and injection procedure.

TABLE I —TOTAL ALKALOIDS OF *Datura stramonium*

Group	Leaves Alkaloids,[a] %	Leaves Difference, %	Stems Alkaloids,[a] %	Stems Difference, %	Roots Alkaloids,[a] %	Roots Difference, %
Control	0 230		0 058		0 071	
Adipic Acid 0 5%	0 263	+14 3	0 073	+25 9	0 127	+78 9
Methylamine HCl 0 5%	0 253	+10 0	0 051	−12 1	0 099	+39.4
Adipic Acid 0 5% and Methylamine HCl 0 5%	0 281	+22 2	0 058	+00 0	0 117	+64 8

[a] Alkaloids calculated as hyoscyamine

The plants were harvested on August 23, 1956 No significant difference was noted in the total weight of the groups receiving treatments compared with the control group The plants were divided into three portions: leaves (including flowering tops), stems, and roots The fresh materials were then transferred immediately into a hot-air circulating dryer and dried at a temperature of approximately 49° The dried material was ground into a No 60 powder and stored in an airtight container until subsequent alkaloid analyses

The Total Alkaloid Assay.—The dried leaves, stems, and roots were assayed for total alkaloids according to the Witt and Youngken method (8) modified by using chloroform in place of benzene as the immiscible solvent This study indicated, in general, that adipic acid and/or methylamine in the concentration of 0 5 per cent increased the total alkaloid yield (calculated as hyoscyamine) in the various plant organs (see Table I and Fig 1) The figures represent the average of four assays for each group

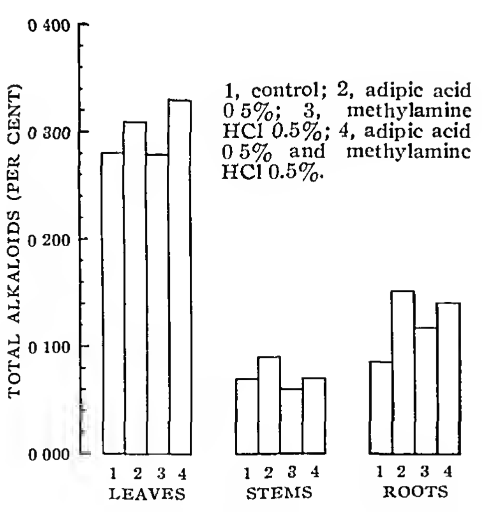

Fig. 1—Total alkaloid yield of *Datura stramonium.*

DISCUSSION

The data on the total alkaloids (Table I and Fig. 1) revealed that adipic acid in the concentration of 0 5 per cent stimulated alkaloid formation in the leaves, stems, and roots by 14, 26, and 79%, respectively. Methylamine in the same concentration increased alkaloid production in the leaves and roots by 10 and 39%, while a decrease of 12% in total alkaloids was found in the stems The combination of adipic acid and methylamine increased total alkaloids in the leaves and roots by 22 and 65%, while no change in alkaloid formation in the stems was observed In general, increased production of total alkaloids in the various plant organs resulted from the treatments Adipic acid appeared to have the greatest influence. Mothes (9) has suggested that diketoadipic acid was a precursor of the *Solanaceous* alkaloids This study showed that adipic acid stimulated alkaloid biosynthesis in *Datura stramonium* and would tend to confirm his work since adipic acid and diketoadipic acid are chemical homologues.

SUMMARY

1. Adipic acid, 0.5 per cent, increased the total alkaloid yield in the leaves, stems, and roots.

2. Methylamine increased total alkaloids in the roots, but there was no great difference in total alkaloid yield in the leaves and stems.

3. The combination of adipic acid and methylamine showed an increase of the total alkaloids in the leaves and roots, but not in the stems.

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Analysis of Phenylephrine and Tetracaine by Filter Paper Chromatography*

By HERBERT SCHRIFTMAN

Experimental preparations containing phenylephrine hydrochloride and tetracaine hydrochloride have been assayed by a paper chromatographic procedure. The results have compared favorably to those obtained by present spectrophotometric and colorimetric methods. Deteriorated samples, obtained from stability studies, have been shown by present methods to contain almost all the phenylephrine hydrochloride as claimed. However, paper chromatographic assays of these same samples have shown the phenylephrine content to be below claim and one to three degradation compounds to be present. For the chromatographic assay, a normal butanol-acetic acid-water (5:1:3) solvent system is employed and a spray reagent containing diazotized p-sulfanilic acid is used to detect the phenolic compounds on the paper. The completed chromatograms are quantitatively analyzed by photoelectric densitometry. Investigations are being conducted to identify the decomposition products.

PHENYLEPHRINE HYDROCHLORIDE, a sympathomimetic amine has been in general use a long time but is becoming available in a growing number of pharmaceutical preparations, containing other active compounds. These complex mixtures present the analyst with additional laborious filtering and extraction steps in conducting successful assays with the conventional colorimetric or spectrophotometric methods. Specifically, one of these methods is Auerbach's colorimetric procedure (1) which is based on the intensity of the azo color produced by coupling phenylephrine with diazotized *p*-nitroaniline. But this reaction only indicates the presence of the hydroxyphenol nucleus in the phenylephrine molecule. Hence, when the various experimental mixtures of phenylephrine hydrochloride in combination with other active compounds are subjected to stability studies, any changes on the side chain would not be detectable by this method and the preparation would apparently still contain all the phenylephrine as claimed. Ellin and Kondritzer (2) presented a colorimetric and spectrophotometric method for analyzing solutions containing phenylephrine hydrochloride and tetracaine hydrochloride. But their color reaction for phenylephrine is also based on the presence of the hydroxyphenol group. They treated the phenylephrine hydrochloride solutions with 15 per cent $HgSO_4$ and then diazotized with 0.1 per cent $NaNO_2$. Phenols and phenolic derivatives are known to react with mercuric salts in the presence of nitrous acid to form intensely colored compounds. The main drawbacks in their procedures were: (*a*) the formation of a precipitate on the addition of the $HgSO_4$ reagent when glycols are present in the solution and (*b*) the additional step in their assay of having to extract any *p*-butylaminobenzoic acid that may be present, as this compound is known to be a hydrolysis product of tetracaine. In the paper partition chromatographic procedure presented here, the extraction and filtering processes are eliminated and separate quantitative analyses of these compounds are possible.

EXPERIMENTAL

Standard mixtures of the hydrochloride salts of tetracaine and phenylephrine in 5 to 25 μg. amounts were spotted and then separated out on Whatman No 1 chromatographic paper sheets by the upper phase of a butanol-acetic acid-water (5.1:3) solvent system, using the descending technique for twenty hours. The tetracaine salt, analogous in structure to alkaloids in that it is a basic nitrogenous compound, might be expected to react to a modified Dragendorff spray reagent, as reported by Munier (3) for detecting various alkaloids that he had separated out on paper. This reagent consisted of a mixture of bismuth subnitrate in glacial acetic acid and KI solution. The reagent was prepared from two solutions: solution 1 contained 0.85 Gm. of bismuth subnitrate dissolved in 40 ml. of water and 10 ml. of glacial acetic acid; solution 2 contained 8 Gm. of KI in 20 ml. of water. Just before use, 5 ml. each of solutions 1 and 2 were mixed with 20 ml. of glacial acetic acid and 100 ml. of water. The tetracaine spots, when sprayed with this reagent, gave the characteristic alkaloid reaction—a red spot on a light orange background. The average R_f value of the tetracaine spots was 0.83. For the separated phenylephrine hydrochloride spots, a general spray reagent for phenols was used, diazotized *p*-sulfanilic acid (4) The diazo reagent was prepared by mixing 20 ml. of a 5% $NaNO_2$ solution with 20 ml of a solution containing 1 Gm. of *p*-

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This work was begun in the Physiology Division of the Chemical Warfare Lab, Army Chemical Center, Md

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The author wishes to express his gratitude to Lt Bobby B Stern and Dr Albert A Kondritzer of the Chemical Warfare Laboratories for their assistance and cooperation, and to Robert Shultz of Wyeth Laboratories for his encouragement and advice.

sulfanilic acid and 15 ml of concd HCl in a liter of distilled water. After spraying with this diazo reagent, the paper was made alkaline with a 5% K_2CO_3 spray. The phenylephrine hydrochloride salts showed up as intense yellow spots whose average R_f value was 0.63. Determination of these R_f values enabled us to cut the developed paper, prior to spraying, so that phenylephrine and tetracaine hydrochloride spots could be analyzed separately. All the colored spots were quantitatively analyzed with a Photovolt Densitometer, Model 525, containing semiautomatic plotting equipment and color filters. Table I shows that for the concentration range studied, the total color density readings are directly proportional to the concentration of the tetracaine and phenylephrine hydrochloride salts. Table II lists the results obtained from the analyses of powdered mixtures of these drugs which had been subjected to stability studies The results obtained by the chromatographic method compare favorably with those obtained by the spectrophotometric and colorimetric method as described by Ellin and Kondritzer (2). However, when solutions of these powders are subjected to heat, these colorless solutions turn brown and paper chromatographic analysis discloses the presence of another phenolic spot at R_f 0.57, as well as other less intense phenolic spots at various R_f values Assays of these deteriorated samples by the Ellin and Kondritzer method indicated that they contained almost all the phenylephrine hydrochloride as claimed. Quantitative paper chromatography of these same samples disclosed that the phenylephrine content was below claim in the range of 12 to 28% depending on the temperature conditions. Table III illustrates some preliminary observations and R_f values of various degradation products of phenylephrine after undergoing accelerated storage conditions. Further studies on the isolation and characterization of the various breakdown products of phenylephrine are under way and will be reported at a later date. Initial absorption spectra of the main breakdown product as well as two other degradation products has been obtained and are compared to the absorption spectra of phenylephrine hydrochloride in Fig. 1 All curves in Fig 1 were obtained on a Beckman Recording Spectrophotometer. The concentration of the phenylephrine hydrochloride solution was 0 05 mg /ml in absolute alcohol The other curves represent eluates of spots of various R_f values from ten papergrams containing ten 200 µg. spots of a degradated solution of 5 mg /ml. of phenylephrine in 5% dextrose (see Table III). The curve for the

TABLE I.—RELATIONSHIP BETWEEN CONCENTRATION AND TOTAL COLOR DENSITIES OF PHENYLEPHRINE HCl AND TETRACAINE HCl SPOTS OBTAINED BY PAPER PARTITION CHROMATOGRAPHY

	µg	Total Color Density = Area under Curve in mm²	Density/ Concn = K
Tetracaine HCl, using a 530 mµ color filter	5	24 0	4 8
	10	48 0	4 8
	15	71 5	4 8
	20	93 5	4 7
	25	118 0	4 7
Average			4 8
Phenylephrine HCl, using a 440 mµ color filter	5	230	46
	10	449	45
	15	680	45
	20	939	47
	25	1119	45
Average			46

TABLE II.—COMPARISON BETWEEN PAPER CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS USED IN THE ANALYSIS OF POWDERS OBTAINED FROM PACKETS STORED UNDER VARYING TEMPERATURE CONDITIONS

	Analysis for Tetracaine HCl, mg		Analysis Phenylephrine HCl, mg	
Samples	C[a]	S	C	S
1—Blank	0	0	0	0
2—Standard	25 0	25 0	25 0	25 0
3—Room Temp.	27 0	27 0	29 0	28 5
4—51°	38 5	38 0	31 5	33 0
5—65°	33 3	33.5	29 6	31 0
6—70°	32.5	31 5	31 5	30 5

[a] C = Chromatographic. S = Spectrophotometric

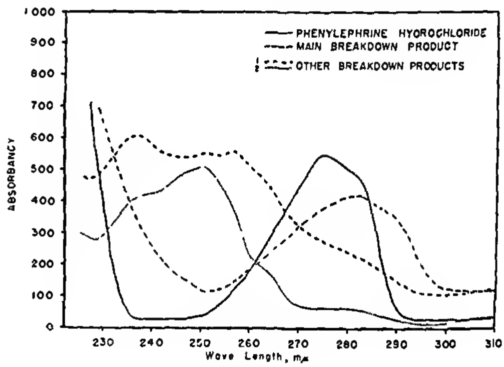

Figure 1.

TABLE III.—R_f VALUES OF PHENYLEPHRINE HYDROCHLORIDE AND ITS DEGRADATION PRODUCTS PRESENT IN DIFFERENT SOLUTIONS, AFTER STORAGE AT 75° C FOR ONE WEEK

		Reagent		
Solution	R_f	Fluorescence[a]	Phenolic[b]	Secondary N[c]
5 mg /ml in distilled water	0 57	+	+	—
	0 64	+	+	+
	0 74	+	+	
	0 86	+	—	
5 mg /ml. in 50% glycerin	0 57	+	—	
	0 59	+	+	—
	0 62	+	+	+
	0 71	+	+	
5 mg /ml. in 5% dextrose	0 26	+	—	
	0 37	+	—	
	0 58	+	+	—
	0 64	+	+	+
	0 75	+	—	

[a] Fluorescence observed under U V light, long-wave type
[b] Diazotized p-sulfanilic acid reagent + 5% K_2CO_3 as described in text
[c] 0 2% ninhydrin in 96% ethanol, containing 2% of acetic acid and 0 5% of cadmium acetate, a positive reaction is the presence of a pink spot (5)

main breakdown product represented eluates of spots at R_f 0 58, while the U. V. spectrum for breakdown products Nos 1 and 2 were obtained from spots at R_f 0 26 and 0 37, respectively In all cases, 35 ml of absolute alcohol was used for eluting the spots from the chromatograms, and then concentrated to 5 ml prior to scanning The reference solution was prepared from a similarly treated blank papergram

SUMMARY

1. A paper chromatographic method is employed for analyzing phenylephrine hydrochloride and tetracaine hydrochloride which compares favorably with the spectrophotometric method.

2. The method presented here for the analysis of phenylephrine HCl and tetracaine HCl is recommended when coloration, foreign matter, or precipitants are present in the solutions.

3. Preliminary studies have been conducted to isolate and identify the degradation compounds of phenylephrine.

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Inhibition of Glucose-6-phosphatase Activity by Hypoglycemic 2-Benzenesulfonamido-5-alkyl-1,3,4-thiadiazoles*

By ROLAND JASMIN and WILLARD JOHNSON

Inhibition of glucose-6-phosphatase (G-6-P-ase) has been considered as a possible mechanism of action of the hypoglycemic sulfonylurea compounds. Experiments have been carried out to ascertain if the hypoglycemic activity of these agents could be correlated with their effect on G-6-P-ase activity. The latter was determined by the method of Cori and Cori (*J. Biol. Chem.*, 199, 661 (1952)). A number of newly synthesized 2-benzenesulfonamido-5-alkylthiadiazoles which inhibited G-6-P-ase activity completely at 2.5 or 5 $\times$ 10^{-3} *M* had negligible hypoglycemic effect, as compared with tolbutamide, which at 8 $\times$ 10^{-3} *M* gave only 50 per cent inhibition and was strongly hypoglycemic. Sulfaethylthiadiazole, an antibacterial sulfonamide with no hypoglycemic activity, inhibited G-6-P-ase activity to the same extent as did tolbutamide. Phenethyldiguanide (PEDG), a nonsulfonylurea compound with marked hypoglycemic activity, failed at 5 $\times$ 10^{-3} *M* concentration to inhibit G-6-P-ase. The evidence indicates that no relationship exists between hypoglycemic activity *in vivo* and G-6-P-ase activity *in vitro*.

THERE IS EXTENSIVE EVIDENCE to support the view that the liver is an important, if not the principal, site of action of the oral diabetic compounds (1–4), the activity of these compounds in the hepatectomized animal notwithstanding (5). Thus, Anderson, *et al.* (1), observed in dogs a 33 per cent decrease in glucose output of the hepatic vein within ten minutes of intravenous tolbutamide administration. A similar effect of tolbutamide was noted by Ashmore, *et al.* (2), in dogs and rats, and by Kibler and Gordon (3) in diabetic patients

The liver is virtually the sole source of blood sugar during the postabsorptive state. Directly involved in the release of glucose to the blood stream is the enzyme, glucose-6-phosphatase, which catalyzes the conversion of glucose-6-phosphate to glucose and inorganic phosphate. Cori and Cori (6) have found this enzyme to be almost completely absent from the livers of human cases of glycogen storage (von Gierke's) disease.

Inhibition of glucose-6-phosphatase has been considered as a possible explanation for the decreased output of glucose by the liver following the administration of hypoglycemic sulfonamides Some investigators have demonstrated an inhibitory effect by tolbutamide and carbutamide under various experimental conditions (7–10) Glucose-6-phosphatase as a primary site of action of these substances has been largely discounted due to the fact that the concentrations required to inhibit the enzyme *in vitro* are much greater than the amounts needed for a hypoglycemic response in the intact animal.

The synthesis of a large series of 2-benzenesulfonamido-5-alkyl-1,3,4-thiadiazoles in our laboratories (11) enabled us to study the relationship between the inhibitory effects of these compounds

* Received June 30, 1958, from the Frank W Horner Ltd , Research Laboratories, Montreal, Quebec

on glucose-6-phosphatase *in vitro* and their hypoglycemic activity. It was considered that, apart from contributing to the elucidation of the mechanism of action of these agents, the establishment of such a correlation would provide a valuable screening method for hypoglycemic agents.

EXPERIMENTAL

Albino mice (20 to 30 Gm.) fed *ad libitum* on Purina Fox Chow were used in these studies. The animals were killed by decapitation, and the livers rapidly removed and placed on ice. Liver homogenates (20% w/v) were prepared in cold distilled water. The contents of each experimental vessel was as follows: 0.5 ml. of 0.02 M glucose 6-phosphate, 0.2 ml. of 0.3 M potassium citrate buffer, pH 6.8, 0.3 ml. of 20% liver homogenate, 1 ml. of glucose 6-phosphatase inhibitor to give a final concentration as indicated in the tables. The control vessel contained 1 ml. of water in place of inhibitor. Incubation was for thirty minutes at 32° with constant shaking. The glucose-6-phosphatase activity was determined by the method of Cori and Cori (6). The release of inorganic phosphate was measured by the method of Fiske and Subba Row (12).

Glucose-6 phosphate, as the barium salt, was purchased from Schwartz Laboratories Inc., and was converted to the sodium salt before using. Neutral solutions of the sodium salts of the sulfonamidothiadiazoles and of Mobenol (tolbutamide, Horner) were used in all studies. The basic structure of the thiadiazole derivatives is shown in Table I.

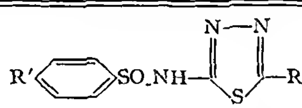
RESULTS AND DISCUSSION

The inhibitory effects of the sulfonamidothiadiazole derivatives and other hypoglycemic agents on mouse liver glucose 6-phosphatase are given in Tables I and II. The relative hypoglycemic activities of these substances in the rabbit, which have been reported by McColl, *et al.* (13), from this laboratory, are roughly indicated in the last column. It should be noted that the dosage employed to obtain the indicated hypoglycemic response bears no relation to the concentration of substance at which glucose-6-phosphate was tested.

It may be seen from Table I that many of the substituted thiadiazoles (compounds, 3, 5, 6, 13, 15, and 17) which inhibited glucose 6-phosphatase completely at 2.5 and 5 × 10^{-3} M had insignificant hypoglycemic activity *in vivo*, as compared with Mobenol (tolbutamide, Horner), which at 5 × $10^{-3}M$ concentration inhibited glucose 6-phosphatase by 37.5% and had strong hypoglycemic activity. The thio analog of Mobenol, devoid of hypoglycemic activity, was slightly more active than Mobenol as an inhibitor of glucose-6-phosphatase. Phenethyldiguanide, which has been shown to be a potent hypoglycemic agent in the normal and alloxanized animal (14) failed completely to inhibit glucose-6 phosphatase, as did glycocyamine (guanidoacetic acid).

Table II provides a comparison of the sulfonamidothiadiazoles in terms of the concentrations required for 50% inhibition of glucose-6-phosphatase. Here it may be seen that compounds 7 and 8, which were found to have potent hypoglycemic activity, inhibited glucose 6-phosphatase to the same extent

TABLE I.—PER CENT INHIBITION OF MOUSE LIVER GLUCOSE-6-PHOSPHATASE



Compd.	Substitution R′	R	Molar Concentration 5 × 10^{-3}	2.5 × 10^{-3}	1.25 × 10^{-3}	Hypoglycemic Activity[a]
			A. Sulfonamidothiadiazoles			
1	CH_3O	*t*-butyl	—	—	20	+++
2	CH_3	*t*-butyl	—	98	32	++
3	C_2H_5O	*t*-butyl	—	100	21	+
4	CH_3O	*n*-butyl	—	100	—	++
5	CH_3	*n*-butyl	—	100	—	+
6	C_2H_5O	*n*-butyl	—	100	—	+
7	CH_3O	iso-butyl	—	—	34	+++
8	CH_3	iso-butyl	—	—	54	+++
9	C_2H_5O	iso-butyl	—	93	—	—
10	CH_3O	*n*-amyl	—	—	29	+++
11	CH_3	*n*-amyl	—	—	98	++
12	CH_3O	iso-amyl	—	—	26	+
13	CH_3	iso-amyl	—	—	100	+
14	CH_3O	iso-propyl	100	—	—	++
15	CH_3	iso-propyl	100	—	—	—
16	CH_3O	*n*-propyl	44	—	—	—
17	CH_3	*n*-propyl	100	—	—	—
			B. Miscellaneous			
Mobenol			37.5	—	—	+++
Thio analog of Mobenol			51.0	—	—	—
Phenethyldiguanide			0.0	—	—	+++
Glycocyamine			0.0	—	—	++

[a] +++ indicates a decrease in blood glucose of 30–40 mg. %, ++ 20–29, +, 10–19, — less than 10.

TABLE II.—CONCENTRATION OF SUBSTANCE REQUIRED FOR 50% INHIBITION OF GLUCOSE-6-PHOSPHATASE

Compd	Sulfonamidothiadiazole R′	R	Moles/ Liter	Hypo-glycemic Activity[a]
1	CH_3O	*t*-butyl	3.0×10^{-3}	+++
2	CH_3	*t*-butyl	2.0×10^{-3}	++
4	CH_3O	*n*-butyl	1.5×10^{-3}	++
7	CH_3O	iso-butyl	2.25×10^{-3}	+++
8	CH_3	iso-butyl	1.5×10^{-3}	+++
11	CH_3	*n*-amyl	6.0×10^{-4}	++
17	CH_3	*n*-propyl	2.0×10^{-3}	−
18	C_2H_5O	*n*-propyl	2.0×10^{-3}	−
19	NH_2 (sulfaethylthiadiazole)	ethyl	8.0×10^{-3}	−
	Mobenol		8.0×10^{-3}	+++

[a] See Table I

as compounds 17 and 18, which have no hypoglycemic activity. Similarly, Mobenol and sulfaethylthiadiazole, a potent antibacterial sulfonamide, each inhibited glucose-6-phosphatase by 50% at 8.0×10^{-3} *M*, while only the former had hypoglycemic activity.

CONCLUSIONS

These results clearly show the complete absence of any correlation between the hypogylcemic effects of these compounds and their glucose-6-phosphatase inhibitory effects *in vivo*. They tend to confirm the observations of other investigators that inhibition of glucose-6-phosphatase is not the principal mechanism by which these compounds exert their effects. That is not to say, however, that inhibition of glucose-6-phosphate *in vivo* does not, in some cases, contribute to the overall hypoglycemic effect.

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The Effect of Nicotine on Hypercholesterolized Cockerels*

By DUANE G. WENZEL, JAMES A. TURNER, and DONALD KISSIL

Cockerels were hypercholesterolized with a 1 per cent cholesterol diet and administered nicotine (2.28 milligrams per kilogram per day) in the drinking water. After sixteen weeks of treatment, the plasma cholesterol and lipid phosphorus levels and the cardiovascular pathology were not significantly different from those of the cholesterol controls.

A NUMBER of recent reports have suggested a possible causal relationship between nicotine and atherosclerosis. Mortality studies reveal a twofold increase in the coronary death rate of smokers over nonsmokers (1). Positive correlation has also been established between the amount of smoking on one hand and both the earlier appearance of coronary occlusion and death from this cause (2). Laboratory studies utilizing rabbits have demonstrated that when nicotine is added to a hypercholesterolemic stimulus, significantly higher plasma cholesterol and lipid phosphorus levels result. The effect is essentially the same whether the cholesterol and nicotine are injected (3) or administered perorally (4). In both of these studies utilizing rabbits a minimal amount of cholesterol corresponding to approximately 0.1 per cent of the diet was utilized.

The purpose of this investigation was to determine whether the chicken is susceptible to the nicotine potentiation of hypercholesterolemia and whether a pathological differential may be established by the use of a normally atherogenic dietary level (1.0 per cent) of cholesterol (5).

EXPERIMENTAL AND DISCUSSION

White English Leghorn cockerels were raised on a diet of Purina Layena® pellets from two days to ten weeks of age. At this time four experimental groups of fourteen birds per group were established. Birds were randomly selected from a group of approximately equal weights. The experimental groups

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were as follows (*a*), control, (*b*), nicotine (2.28 mg./Kg. per day in the drinking water), (*c*), cholesterol (1.0% in the feed), and (*d*), combined nicotine-cholesterol regimens. The daily dose of nicotine was roughly equivalent to the use of two packs of cigarettes by the human (4).

At ten weeks of age the birds were weighed and plasma cholesterol and phospholipid levels determined by methods previously described (4). Determinations were repeated at four-week intervals over the sixteen week experimental period. At twenty-six weeks of age all birds were sacrificed and the hearts and aortas studied for pathological changes.

The control group gained weight at a slightly but not significantly greater rate than the other birds. Figure 1 is a record of the plasma total cholesterol levels and the cholesterol/phospholipid ratios over the sixteen week period. The cholesterol and nicotine cholesterol group values were not significantly different from one another because of rather large variations between birds. These variations were

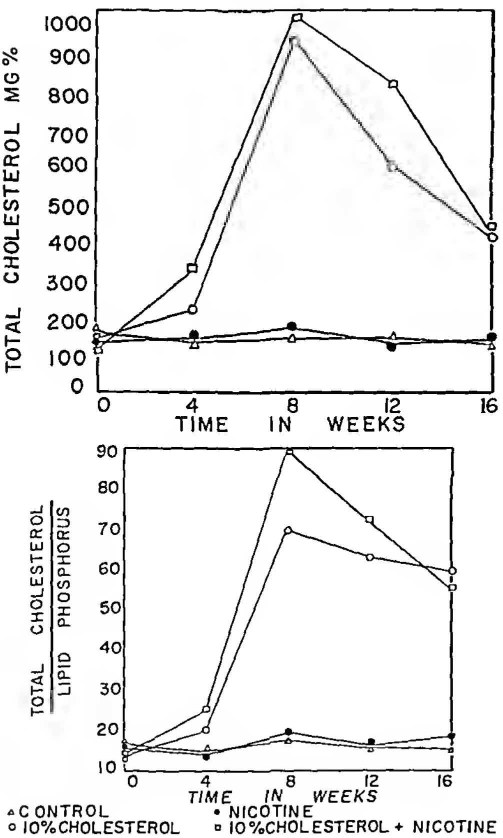

Figure 1

greatest at the eight and twelve week periods when the differences in cholesterol levels and the ratios were most apparent. The effect of nicotine as a hypercholesterolemic or C/P stimulus is at best only suggestive.

The heart and entire aortas were removed from each animal. In each instance the aortas were grossly examined and five sections prepared from the tissues. The first included myocardium, leaflet of mitral valve, and ascending aorta with coronary arteries. The remaining sections consisted of a transverse section of the wall of the aorta, a transverse section of the innominate arteries, a transverse section of the left pulmonary artery, and an opened transverse section of the terminal aorta. In each instance H and E, toluidine blue, and von Kossa's stain were used. In addition, a separate block of the myocardium, aortic valve, coronary artery, and ascending aorta were studied by means of a frozen section with a fat stain.

The gross and microscopic studies of the control and nicotine groups revealed essentially no changes. Three birds of the cholesterol group demonstrated small yellow plaques in the aorta, none of which was greater than 2 mm. in diameter. Upon microscopic examination the plaques were found to consist of slightly thickened intima with a myxomatous, edematous appearing fibrous tissue forming the main component. No other significant changes were noted in the cholesterol group.

Three birds of the nicotine cholesterol group also demonstrated plaques in the aorta. Microscopic examination revealed an appreciable increase of fatty material in the thickened intima, particularly in the proximal aorta and aortic valve. There were occasional macrophages filled with a foamy material. The coronary vessels were essentially normal except for minimal thickening.

The fact that minimal pathologic changes observed in both cholesterol groups may indicate that either the particular strain of chicken employed possessed minimal atherogenic susceptibility or that spontaneous regression of the lesions had occurred. Evidence for the latter possibility may be the fall in cholesterol and C/P ratios at eighteen weeks of age. As earlier studies with the rabbit had established a marked effect of nicotine on blood cholesterol levels with a minimum (0.1%) diet of cholesterol, it is possible that the higher dose (1.0%) used in the study obscured any nicotine effect.

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The Analysis of Phenothiazine Derivatives with Tranquilizing Properties*

By JOHN MILNE

Nonaqueous assay methods have been developed for some of the more recently available phenothiazine derivatives with tranquilizing properties. These methods have been compared to Blazek and Stejskal's gravimetric procedure which has been successfully applied to these drugs in this laboratory as well as to the control procedures used by the pharmaceutical manufacturers. For purposes of nonaqueous titrimetry, the drugs studied may be divided into two groups by virtue of the monobasic or dibasic character of their N bonded side chain. The monobasic drugs are titrated in acetone and 2:1 hexane:acetone while the dibasic compounds which carry a piperazine ring in the side chain are titrated in 2:1 benzene:nitromethane. The differential titration of both the salts and bases of this last group in different solvent systems was studied before the benzene:nitromethane system was chosen and the results of this investigation are included.

With the advent of chlorpromazine and other similar phenothiazine derivatives with tranquilizing properties a need for analytical control procedures has arisen. Several methods have been introduced for these drugs and chemically similar antihistamines.

Colorimetry, involving both the reineckates (1) and the picrates (2), has been employed in the assay of these drugs. Brodie (3) complexed organic bases with acidic indicators and measured the colors formed, Porter and Silber (4) applied this technique to diethazine, and Haley and Bassin (5) utilized it for promethazine. Fossoul (6) used the color produced by the interaction of ammonium persulfate with promethazine, and Dubost and Pascal (7) determined chlorpromazine by measuring the color which it produces with concentrated sulfuric acid.

Ultraviolet spectrophotometry in water forms the basis of the United States Pharmacopeia procedure (8) for chlorpromazine tablets. This technique has also been applied to chlorpromazine, mepazine, and promethazine by Meyer (9) with ether and ethanol as solvents.

Several titration procedures have been reported among which are the argentometric titration (1) of the hydrochloride salts and nonaqueous titration of the sodium tetraphenylborates of both chlorpromazine and diethazine (1). Nonaqueous techniques have also been applied to promethazine hydrochloride by Kleckner and Osol (10), and to promazine and chlorpromazine by Milne and Chatten (11). Sandri (12) has determined promethazine, chlorpromazine, and diethazine by a bromination technique involving back titration of the excess bromide. The 1958 British Pharmacopoeia procedure for chlorpromazine is an acid-base back titration method (13) and the United States Pharmacopeia method for crystalline chlorpromazine is a nonaqueous titration procedure in glacial acetic acid (8).

Blazek (15) has simplified the original gravimetric method with silicotungstic acid which was introduced by Blazek and Stejskal (14). He has employed titration techniques with polarographic detection of the excess tungstate at the end point (15). Diethazine, chlorpromazine, and promethazine have been determined this way.

It was the purpose of this investigation to compare existing methods with the nonaqueous procedures developed in this laboratory, in an attempt to find suitable assays for these phenothiazine derivatives and their pharmaceutical preparations.

EXPERIMENTAL

Reagents.—Glacial acetic acid, A. C. S.; benzene, A. C. S.; chloroform, A. C. S.; acetone, A. C. S.; methanol, A. C. S.; ethanol, 95%; dioxane, Eastman Kodak; hexane, B. D. H. laboratory reagent; dimethylformamide, Anachemia, b. p. 152–154°; nitromethane, B. D. H. laboratory chemical or Eastman Kodak, practical grade, redistilled, b. p. 100–101°; 0.05 N perchloric acid in dioxane standardized against potassium acid phthalate, A. C. S., in glacial acetic acid with crystal violet as indicator; 0.05 N potassium hydroxide in methanol standardized against potassium acid phthalate, A. C. S., in 1:1 water-methanol with phenolphthalein as indicator; 0.1 N sodium methoxide in methanol standardized against benzoic acid, U. S. P., in dimethylformamide with thymol blue as indicator; 1% and 5% dimethyl yellow in chloroform; 1% tropaeolin 00 in methanol; 1% thymol blue in methanol; 6% mercuric acetate in glacial acetic

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The author wishes to express his appreciation for the helpful discussions with L. G. Chatten and his thanks to G. Morris for the figure.

acid, 10% silicotungstic acid solution 40% potassium hydroxide solution, sodium sulfate, A C S

Apparatus —Fisher titrimeter, model No 9 3111A, and Beckman pH meter, model G, with glass indicator electrode and sleeve type calomel reference electrode, 10 ml semimicro buret measuring to 0 02 ml

Nonaqueous Procedures with Crystalline Materials[1]

Dibasic Drugs —Prochlorperazine dimaleate (1 methyl 4 [3 (2 chloro 10 phenothiazinyl) propyl] piperazine dimaleate), thiopropazate dihydrochloride (1-(2 acetoxyethyl) 4 [3 (2 chloro 10 phenothiazinyl) propyl]-piperazine dihydrochloride), Perphenazine (1 (2 hydroxyethyl) 4 [3 (2 chloro 10 phenothiazinyl) propyl] piperazine)

An accurately known quantity (approx 50 mg) of the base is titrated in 2 1 benzene nitromethane with total volume of about 150 ml using perchloric acid in dioxane as titrant and 3 drops of tropaeolin 00 solution as indicator The indicator color change is from yellow through amber to pink violet or complete disappearance of the amber hue Where the starting material is a salt the titration mixture may be obtained by extraction of an aqueous basic solution of the salt with six 15 ml portions of benzene and subsequent addition of nitromethane A minimum of potassium hydroxide solution should be employed while the aqueous solution should be nearly saturated with sodium sulfate in order to prevent emulsion

The acid moiety of thiopropazate dihydrochloride may be titrated in chloroform (50 mg crystalline material in 60 ml solvent) with potassium hydroxide in methanol, or in dimethylformamide (150 mg crystalline material in 50 ml solvent) with potassium methoxide in methanol Three drops of thymol blue indicator solution is used and the color changes are, respectively, yellow through green to full blue, and yellow to gray green or complete disappearance of the yellow hue

Monobasic Drugs —Mepazine hydrochloride monohydrate (N methylpiperidyl (3) methyl phenothiazine hydrochloride monohydrate), acepromazine maleate (10 (3 dimethylaminopropyl) 2 (acetyl) phenothiazine maleate), triflupromazine hydrochloride (10 (3 dimethyl aminopropyl) 2 (trifluoromethyl) phenothiazine hydrochloride)

An accurately known quantity of the salt (approx 70 mg) is dissolved in 50 ml acetone and, in the case of hydrochloride salts, 0 5 ml mercuric acetate solution is added With mepazine and triflupromazine, where 2 drops of 1% dimethyl yellow solution are added, the end point color change is from yellow to orange, whereas with acepromazine, where 2 drops of 5% dimethyl yellow solution are used, the end point color change is from orange yellow to the first full orange color Perchloric acid in dioxane is the titrant in all instances

Mepazine hydrochloride is titrated as the base in 2 1 hexane acetone by extraction of the salt in the same manner as that employed with the dibasic drugs but four 20 ml portions of hexane are used and acetone is subsequently added The procedure then follows exactly that used for the salt

Gravimetric Procedures with Crystalline Materials

This procedure is applicable to all the above mentioned tranquilizing drugs except prochlorperazine dimaleate and follows the original technique of Blazek and Stejskal (14)

Accurately weigh approx 0 06 M E (20 mg with monobasic drugs and 10 mg with dibasic drugs) of the compound into a beaker containing 20 ml distilled water and 1 ml concentrated hydrochloric acid, warm the solution to 70° and add, dropwise, 8 ml silicotungstic acid solution Collect the precipitate in a tared, sintered glass crucible, washing with 50 ml distilled water, and dry to constant weight

Nonaqueous Procedures with Pharmaceutical Preparations

Prochlorperazine Dimaleate Tablets —Weigh and powder 20 tablets Transfer an accurately weighed quantity of the tablets equivalent to 50 mg prochlorperazine into a 250 ml separatory funnel containing 150 ml distilled water, 0 2 ml potassium hydroxide solution and approx 3 Gm sodium sulfate Proceed as in crystalline prochlorperazine dimaleate method extracting with six 15 ml portions benzene

Suppositories —Dissolve 20 suppositories in benzene contained in a 250 ml volumetric flask and make up to the mark with the same solvent Take an aliquot of 40 ml , add 20 ml nitromethane, 1 drop tropaeolin 00 indicator solution, and titrate with perchloric acid in dioxane The end point is the same as that observed with the crystalline prochlorperazine dimaleate determination

Thiopropazate Dihydrochloride Tablets.—Proceed as directed for the prochlorperazine dimaleate tablets determination

Mepazine Hydrochloride Monohydrate, Acepromazine Maleate, and Triflupromazine Hydrochloride Tablets —Weigh and powder 20 tablets Accurately weigh a quantity of tablet mass equivalent to about 70 mg of the drug and extract with 50 ml of acetone for ten minutes, employing an electromagnetic stirrer Filter through a fine sintered glass funnel with the aid of suction, washing the beaker and residue with 30 ml acetone Proceed as directed in the determination of the crystalline materials for each particular compound beginning after the dissolution of the pure drug in acetone

Mepazine Ampuls —Empty the contents of 10 ampuls into a beaker and pipet a quantity of ampul solution equivalent to about 70 mg mepazine hydrochloride into a 125 ml separatory funnel containing 25 ml distilled water, 0 2 ml potassium hydroxide solution, and 1 Gm sodium sulfate Proceed as directed in the crystalline mepazine hydrochloride monohydrate determination as the base extracting with four 20 ml portions of hexane

Gravimetric Procedures with Pharmaceutical Preparations

Acepromazine, Perphenazine, and Triflupromazine Tablets —Weigh and powder 20 tablets Weigh a quantity of the tablet mass equivalent to about 0 15 milliequivalents of active ingredient and

[1] In all nonaqueous procedures the blank for the particular solvent system used should be determined using the same indicator and end point color change as described in the procedures themselves

extract with 25 ml ethanol for ten minutes employing an electromagnetic stirrer Filter through a fine sintered-glass funnel with the aid of suction, washing the beaker and residue with 30 ml ethanol Evaporate the ethanol and redissolve the residue in 10 ml distilled water containing 0 5 ml concentrated hydrochloric acid Transfer to a 50 ml volumetric flask with the aid of hydrochloric acid solution of the same concentration and make up to the mark with the same acid solution Take a 20-ml aliquot, filter if necessary, and proceed as directed for the determination of the crystalline material beginning at "warm the solution "

Acepromazine Ampul and Drop Solutions.—Take an aliquot equivalent to 0 06 M E of active ingredient from the solution of 10 ampuls or the drops solution, make up to 20 ml, and add 1 ml concentrated hydrochloric acid Proceed as directed for the determination of the crystalline material beginning at "Warm the solution "

DISCUSSION

The phenothiazine derivatives investigated in this work may be classified by their monobasic or dibasic character The monobasic drugs triflupromazine hydrochloride, mepazine hydrochloride monohydrate, and acepromazine maleate were assayed similarly to the hydrochlorides of chlorpromazine and promazine as outlined in a previous publication from this laboratory (11) Dimethyl yellow indicator, however, was used in place of methyl red Mercuric acetate was employed with the hydrochloride salts in both instances Assay results and standard deviations for crystalline drugs are given in Table I and those for pharmaceutical forms in Table II

For solubility reasons, extraction from aqueous basic medium was employed with prochlorperazine dimaleate and thiopropazate dihydrochloride while direct dissolution in the extraction solvent was used for perphenazine base The water immiscible extraction solvents chosen were benzene, chloroform, and hexane and for potentiometric titration purposes these were mixed with nitromethane, acetonitrile, and acetone While differential titration curves were obtained in all instances except hexane nitromethane and hexane acetonitrile which were immiscible combinations, the second potential break was sharp enough only in the hexane acetone and benzene nitromethane systems to warrant further investigation Only the latter system was used in this work, the hexane acetone combination being the subject for future investigation Tropaeolin 00, the indicator used by Yokoyama and Chatten (18) in the benzene nitromethane formic acid system was found to be satisfactory for the titration of these dibasic drugs

Pifer and Wollish (17) have reported that the addition of an aprotic solvent to a polar solvent sharpens the end point In the hexane acetone and benzene nitromethane combinations such addition appears to improve the second potential break but at the expense of the first end point in both instances The separate components of the benzene nitromethane system have been employed before for the titration of bases by various workers (19, 20, 21) However, Chatten, Pernarowski, and Levi (22) have reported a disadvantage with nitromethane Apparently amines with pKb values less than 11 display two potential breaks when titrated in nitromethane They have shown that the formation of a nitromethane addition product with the amine is possible and postulate that its formation accounts for the second potential break and limited success in the titration of amines reported by Fritz and Fulda (20) Although it has been observed here that acepromazine and mepazine display two potential breaks when titrated in benzene nitromethane (2 1), no such phenomenon was encountered upon titration of the dibasic drugs in this system nor have Ciaccio, *et al* (21), reported any trouble in their work with nitromethane However, as an added precaution against erratic recoveries due to such interference, the second and last potential break was used for the calculation of recoveries Tropaeolin 00 changes color at this potential break and the curve and color changes depicted in Fig 1 are representative of the titrations of all the dibasic drugs in benzene nitromethane

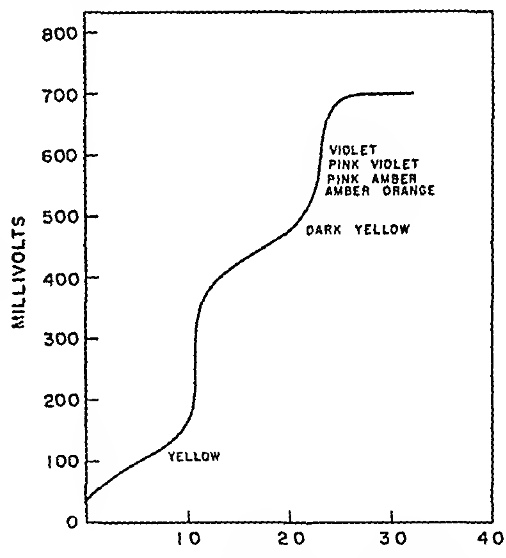

Fig 1—Titration of prochlorperazine base from crystalline prochlorperazine dimaleate in a 2 1 mixture of benzene nitromethane using the Beckman pH meter

Although the benzene nitromethane system was chosen specifically for the assay of the pharmaceutical forms of the dibasic drugs and its application to crystalline drug was successful, its use was limited with the manufactured product Prochlorperazine dimaleate and thiopropazate dihydrochloride were successfully determined in tablets but thiopropazate dihydrochloride gave erratic recoveries, indicated by a large standard deviation (see Table II) The recoveries by the procedure for perphenazine tablets were both low and erratic Upon investigation of the tablet formulation, the activated charcoal, present as an excipient, was shown to be the cause The results for all the crystalline dibasic drugs and their pharmaceutical forms along with standard deviations are given in Tables I and II, respectively

TABLE I —RESULTS OF THE ANALYSES OF CRYSTALLINE DRUGS

Crystalline Form of Drug	Mol Wt	Gravimetric Conversion Factor	Gravimetric Recovery, %	Nonaqueous Recovery, %	Nonaqueous Standard Deviation	Control Procedure Recovery, %
Triflupromazine hydrochloride	388.88	0.3658	99.52	99.20	0.425	
Acepromazine maleate	444.52	0.4267	99.68	100.06	0.144	100.6
Mepazine hydrochloride monohydrate	364.93	0.3574	100.80	99.14 (as salt)	0.245	
		0.3514 (with one mole water)	99.80	99.02 (as base)	0.446	
Prochlorperazine dimaleate	606.07			98.96	0.253	100.4
Perphenazine	403.99	0.2213	98.50	99.50	0.364	
Thiopropazate dihydrochloride	519.94	0.2847	98.11	98.06	0.447	98.98 (in $CHCl_3$) 98.05 (in DMF)

TABLE II —RESULTS OF THE ANALYSES OF PHARMACEUTICAL FORMS

Pharmaceutical Form	Brand Name	State of Active Ingredient	Nonaqueous Recovery, %	Nonaqueous Standard Deviation	Gravimetric Recovery, %	Control Procedure Recovery, %
Triflupromazine tablet	Vesprin	Hydrochloride	99.13	0.655	98.20	99.38
Acepromazine tablet	Plegicil	Maleate	97.01	0.590	97.84	108.00
Acepromazine drops		Maleate	98.36		103.2	111.00
Acepromazine ampul		Maleate	95.23		98.16	92.00
Mepazine tablet	Pacatal	Hydrochloride	100.18	0.610		98.96
Mepazine ampul		Acetate	102.10	0.150		99.04
Prochlorperazine tablet	Stemetil	Dimaleate	96.63	0.680		97.01
Prochlorperazine suppository		Free base	102.10	0.162		
Perphenazine tablet	Trilafon	Free base			103.2	102.55
Thiopropazate tablet	Dartal	Dihydrochloride	99.67	1.62		98.24

Both the Fisher titrimeter and Beckman pH meter with glass-sleeve type calomel electrode combination were employed and perchloric acid in dioxane was the titrant in all instances Where the titration solvent was water miscible, leakage of the salt bridge solution from the calomel electrode caused the potential and indicator color change to reverse Replacement of the aqueous salt bridge with a potassium chloride saturated solution of the titration solvents gave erratic results and slow response. The silver-silver chloride reference electrode was tried but it afforded a confined potentiometric range less than one half the range offered by the calomel electrode and could only be used for the titration of the monobasic drugs. However, it was found that the application of grease to the sleeve of the calomel electrode prevented leakage and consequently allowed its use in all cases.

The Blazek and Stejskal gravimetric procedure was applied with success to all the crystalline compounds except prochlorperazine dimaleate. The structure of all silicotungstic acid precipitates was assumed to be four equivalents of nitrogen contributed by the drug to one equivalent of silicon contributed by the silicotungstic acid Table I shows the value of the gravimetric conversion factor used for each precipitate for conversion to the quantity of crystalline drug originally present in the form used, along with percentage recoveries of pure drug calculated by use of these constants With the constant for thiopropazate it was assumed that the drug hydrolyses completely to perphenazine, proof of which is indicated by comparison of the recoveries using this constant with those arrived at by other methods Two constants and percentage recoveries are given for mepazine, the first assuming the precipitate to contain one mole of water as does the hydrochloride salt, the second assuming the absence of water. By comparison with nonaqueous results, the first constant appears to give recoveries closer to the correct figure This gravimetric procedure has been applied to the following pharmaceutical preparations; triflupromazine tablets, acepromazine tablets, ampuls and drops, and perphenazine tablets The results appear in Table II

The control methods which were developed by the pharmaceutical manufacturers for their products were also investigated and compared to the other procedures In all instances but one, prochlorperazine suppositories, at least two methods of

assay, selected from nonaqueous, gravimetric, and control procedures were performed in order to verify results.

Two control procedures have been developed by the manufacturers of prochlorperazine (23) for the determination of crystalline prochlorperazine dimaleate and its pharmaceutical preparations; (*a*) nonaqueous titration of the acid moiety (maleic acid) in dimethylformamide with sodium methoxide in methanol as titrant and thymol blue as indicator and (*b*) nonaqueous titration of the drug in glacial acetic acid with perchloric acid as titrant and α-naphthol benzein as indicator. Neither with the pure substance nor the pharmaceutical forms was it possible in this laboratory to obtain a sharp end point in the former procedure and the accuracy of the method was doubted for this reason. However, results submitted by the manufacturer and those obtained with the same lot of tablets by the nonaqueous method developed in this laboratory compared favorably (see Table II). The titration in glacial acetic acid was limited to pure drug only and the results are given in Table I.

In order to verify the results of the nonaqueous procedure developed here for thiopropazate dihydrochloride using the benzene:nitromethane solvent system, two additional nonaqueous procedures were developed and used, both of which involve the titration of the acid moiety (hydrochloric acid) of thiopropazate dihydrochloride. In the first instance, the drug is titrated in dimethylformamide with sodium methoxide in methanol while, in the second, the solvent is chloroform and the titrant, potassium hydroxide in methanol. When the potentiometric titration curves were taken, differential titrations were observed and the indicator, thymol blue, in both instances changed at the second millivolt maximum. The chloroform curve could not be detected initially because the meter readings were very unsteady until sufficient methanol from the titrant had been added. Table I gives the recoveries by both methods.

An attempt was made to apply both of these procedures to thiopropazate tablets but with little success. The dimethylformamide titration did not give a sharp indicator color change or potentiometric break and the chloroform extraction and titration gave low results. Tablet formulations were made up and certain excipients eliminated in order to determine the cause of the low recoveries in the chloroform titration and it was found that the sodium sulfate, which was present in large quantity, was the interfering ingredient. Since no acidic excipients were present in the tablet formulation, it was felt that extraction and titration in chloroform:methanol might be possible. However, low recoveries were still obtained with the tablets and when crystalline product was titrated, the second potential break and indicator color change deteriorated with the increased proportion of methanol. In the control procedure submitted by the company (24), the compound is determined by the potentiometric titration of the ionic chloride with silver nitrate using the glass-silver electrode combination. The results of this procedure with tablets are given in Table II.

Titration of crystalline thiopropazate dihydrochloride as the acetate in chloroform:nitromethane was also tried. Although no sharp potential breaks resulted and thus the method could not be used, it is interesting to note that a differential titration was obtained. Ciaccio, *et al.* (21), have explained the differential titration of the free bases of piperazine derivatives by the decreased strength of the second nitrogen after neutralization of the first. They explain that the presence of a proton on the neutralized nitrogen lessens the attraction of a proton to the remaining nitrogen. The explanation may be used to account for the differential titration of the acetate in chloroform:nitromethane. The differential acid moiety titrations in dimethylformamide and chloroform may be accounted for similarly by the enhanced proton affinity of the basic nitrogen carrying the untitrated hydrochloride group after removal (by neutralization) of the hydrochloric acid from the other nitrogen. Ciaccio, *et al.* (21), noted that disubstituted piperazine bases displayed only one potentiometric break in glacial acetic acid when titrated with perchloric acid in acetic acid. It has been found that thiopropazate acetate, formed from the hydrochloride with mercuric acetate, acts similarly when titrated with perchloric acid in dioxane. Because perchloric acid is sufficiently strong to partially titrate thiopropazate dihydrochloride, an attempt to titrate the basic excipients before addition of mercuric acetate was unsuccessful.

The control procedure for perphenazine is an ultraviolet spectrophotometric procedure with methanol as solvent (25). Comparisons with a standard solution are read at about 257 mμ. Mepazine is determined similarly with ethanol as solvent for tablets (water for ampuls) and readings are taken at about 254 mμ (26). The results of these procedures as applied to commercial preparations are given in Table II.

For control purposes crystalline acepromazine maleate was determined in glacial acetic acid with perchloric acid as titrant and crystal violet as indicator (27). The results of this assay are given in Table I. However, the commercial forms of this drug were determined by a colorimetric procedure using a sulfuric acid-bromine water reagent (27). Erratic results were obtained by this method even within the readings of the standard solutions. The results of this technique are given in Table II also. Because of the erratic nature of all the readings taken in this procedure and the low recoveries obtained by nonaqueous titrimetry for the aqueous preparations of both pure drug and commercial products, it was felt that the gravimetric recoveries were the most accurate and are the accepted results for the aqueous preparations. The reliability of the gravimetric procedure is also borne out by the fact that the gravimetric and nonaqueous results agree well for both the tablets and crystalline drug.

The control method for triflupromazine tablets is also an ultraviolet spectrophotometric procedure (28) similar to that given for chlorpromazine in the U. S. P. XV supplement (8). Results of this procedure are also given in Table II.

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Some Aspects of the Central Nervous System Activity of a Series of Urazole Derivatives*

By C. L. MITCHELL,† H. H. KEASLING, and E. G. GROSS

The neurotoxicity, antielectroshock, anti-Metrazol, and antistrychnine activities of a series of urazole compounds have been determined in mice. The compounds were relatively short acting and nontoxic. In general, the members of the series were found to be highly specific against maximal Metrazol seizures. Variations in the substituted nitrogen groups yield compounds possessing anti-Metrazol, antistrychnine, convulsant, and depressant type actions.

THE SIMILARITY of the urazole and hydantoin nucleus prompted the present investigation of the C. N. S. activity of a series of substituted urazoles. The structures of the derviatives studied are detailed in Table I. The compounds were administered to mice and their observable effects were noted. In addition, their antielectroshock, anti-Metrazol, and antistrychnine activities were tested. This report details the results of these experiments and some observations with regard to the structure-activity-relationship of their C. N. S. actions.

EXPERIMENTAL

Adult male albino mice obtained from the Carworth Farms (CF No. 1 strain) were maintained on Purina laboratory chow and allowed free access to food and water except during the test period. The mice were used for one experiment only. The test procedures employed were: (*a*) the supramaximal electroshock seizure technique, M E. S, of Toman, *et al.* (1), in which alternating current, 50 ma. of 0 2 second duration was delivered through Spiegel corneal electrodes (2) from an electroshock apparatus constructed according to the design of Woodbury and Davenport (3); (*b*) the maximal Metrazol seizure test, M. M S. of Goodman, *et al.* (4), in which Metrazol,[1] 38 mg /Kg, was injected intravenously. The end points for both tests were the abolition of the tonic extensor components of the seizure pattern; and (*c*) ability to prevent death from strychnine, in which 2 5 mg /Kg of strychnine was injected intraperitoneally (5)

All compounds were suspended in 10% gum acacia for oral administration The convulsant stimulus was originally delivered one hour following urazole administration but early in the testing at 1 Gm./Kg it was noted that the compounds were rapidly absorbed and of short duration. Therefore, subsequent tests at 1 Gm /Kg and all lower doses were determined thirty minutes after dosage Phenobarbital, trimethadione,[2] and diphenylhydantoin[3] were tested at the optimum times suggested by Swinyard, *et al.* (6). Those compounds exhibiting protection at 1 Gm /Kg from electroshock and/or Metrazol were then administered in dosages of 300, 100, and 30 mg /Kg until less than 50% of the animals were protected.

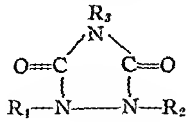
Acute neurotoxicity (NT) was determined by the method of Swinyard, *et al.* (6). The following end points were used: (*a*) positional sense test, (*b*) righting test, (*c*) gait and stance test, (*d*) muscle tone test, and (*e*) equilibrium test Neurotoxicity was recorded when an alteration in response to the above procedures was observed. The mice were examined at fifteen minute intervals for at least one hour and thereafter until two successive examinations indicated no change, or until a decrease in the number of mice exhibiting neurological symptoms was seen.

The 50% neurotoxic dose (TD_{50}) and the 50%

* Received July 28, 1958, from the Department of Pharmacology, College of Medicine, State University of Iowa. Iowa City.
† Smit [illegible] The material [illegible] ate Coll [illegible] ment of [illegible] e compou [illegible] ported [illegible] N J.

[1] The Metrazol was kindly supplied through the courtesy of Dr R O Hauck, Knoll Pharmaceutical Co
[2] The trimethadione was kindly supplied through the courtesy of Dr R K Richards, Abbott Laboratories
[3] The diphenylhydantoin was kindly supplied through the courtesy of Dr Graham Chen, Parke, Davis and Co

TABLE I.—STRUCTURES OF URAZOLE DERIVATIVES



UR No.	R_1	R_2	R_3	UR No.	R_1	R_2	R_3
4	H	H	H	40	$CH_2C(=O)—OC_2H_5$	θ	H
5	H	H	CH_3	17	<S>	<S>	H
7	H	CH_3	H	20	<S>	θ	H
37	H	H	NH_2	24	<S>	<S>	n—C_3
1	H	θ	H	29	<S>	θ	n—C_3
3	H	θ	CH_3	42	<S>	θ	CH_3
8	CH_3	θ	H	44	<S>	θ	CH_2CH_2OH
9	CH_3	θ	CH_3	15	CH_2θ	θ	H
46	CH_3	θ	CH_2CH_2OH	30	CH_2θ	θ	n—C_3
47	CH_3	θ	n—C_3	31	CH_2θ	θ	CH_2θ
10	n—C_3	θ	H	43	CH_2θ	θ	CH_3
12	iso-C_5	θ	H	45	CH_2θ	θ	CH_2CH_2OH
23	n—C_5	θ	H	2	θ	θ	H
25	C_2	θ	H	6	θ	θ	CH_3
26	n-C_3	θ	n-C_3	11	θ	θ	iso-C_5
27	iso-C_5	θ	n-C_3	13	θ	θ	n-C_3
28	iso-C_4	θ	H	14	θ	θ	θ
49	iso-C_3	θ	H	16	θ	θ	CH_2CHCH_2 OH OH
50	iso-C_3	θ	CH_3	18	θ	θ	CH_2CH_2OH
51	iso-C_3	θ	n-C_3	19	θ	θ	CH_2CH_2Cl
52	iso-C_3	θ	iso-C_3	21	θ	θ	C_2
53	iso-C_3	θ	CH_2CH_2OH	22	θ	θ	n-C_5
34	iso-C_3	iso-C_3	H	32	θ	θ	$C(=O)—OC_2H_5$
35	iso-C_3	iso-C_3	iso-C_3	33	θ	θ	$CH_2C(=O)—OC_2H_5$
36	iso-C_3	iso-C_3	n-C_3	39	θ	θ	$CH_2C(=O)—NH_2$
38	$C(=O)—OC_2H_5$	θ	$C(=O)—OC_2H_5$	41	θ	θ	$CH_2C(=O)—ONa$

effective dose (ED_{50}) of the urazole compounds were determined thirty minutes after the administration of the drug. Similar tests for trimethadione, phenobarbital, and diphenylhydantoin were determined at one and one-half hours, two hours, and three hours, respectively. The TD_{50} or ED_{50} was obtained by plotting the effect of at least 3 dose levels yielding between 10 and 90% effect on log-probit graph paper and fitting the points with a straight line. At least 10 mice were utilized in determining each point. The computations for fitting the probit-log dose regression line, the determination of the TD_{50}, ED_{50}, protective index (P. I.), ratio of ED_{50} M. E. S./ED_{50} M. M. S., and the 95% confidence interval were done according to the method of Finney (7).

The time of peak anticonvulsant effect and duration of action of one compound (UR 20) was determined by the M. M. S. test. An approximate ED_{60} of UR 20 was administered to 6 groups of 10 animals. The respective groups of mice were then injected with Metrazol at fifteen, thirty, forty-five, sixty, ninety, and one hundred and twenty minutes.

The sleeping time produced by two compounds (UR Nos 12 and 23) was compared with hexobarbital. Sleeping time was recorded as the time interval in which the righting reflex of the mice was absent. The effect of two convulsant compounds (Nos. 35 and 36) upon the sleeping time of hexobarbital was also determined. The convulsant compounds were administered orally, two minutes prior to the i. p. injection of hexobarbital. The 95% confidence intervals were calculated according to the method of Snedecor (8).

RESULTS

In general, the compounds were relatively nontoxic. No mice were killed at 1 Gm./Kg. with the exception of UR Nos. 34, 35, and 36 which were lethal convulsants (*vide infra*), UR Nos. 12 and 23 which produced stimulation followed by profound depression (*vide infra*), and UR No. 10. The predominant neurotoxic effect was ataxia. Flaccid paralysis was noted following administration of UR 8.

The anticonvulsant activities are detailed in Table II. Our data for phenobarbital (TD_{50}, 60 mg./Kg., ED_{50} M. E. S., 21 mg./Kg.) and diphenylhydantoin

TABLE II —ANTICONVULSANT ACTIVITY OF URAZOLES[a, b, c]

UR No.	Test	1,000	300	100	30	UR No	1,000	300	100	30
8	NT[d]	7/20	0/10				11/20	3/10	0/10	
8	MES[e]	7/10	4/10			20	8/10	7/10	0/10	
8	MMS[f]	5/5	5/5	0/10			5/6	8/10	6/10	3/10
9	NT	4/10	0/10				0/20	0/10		
9	MES	9/10	0/10			42	0/10			
9	MMS	5/5	0/10				7/10	0/8		
46	NT	2/20	0/20	0/10			1/20	0/10		
46	MES	10/10	0/10			44	0/10			
46	MMS	10/10	4/7	1/8			6/10	2/10		
47	NT	6/20	0/20				0/20	0/10		
47	MES	6/10	0/10			30	5/9	0/10		
47	MMS	10/10	3/9				5/8	0/10		
10	NT	20/20	9/10	1/10			0/20	0/10		
10	MES	10/10	10/10	2/10		43	0/10			
10	MMS	5/5	10/10	10/10	2/10		5/8	0/10		
25	NT	20/20	5/10	0/10			0/20	0/10	0/10	
25	MES	10/10	7/10	0/10		45	5/10	0/10		
25	MMS	5/5	10/10	8/8	2/10		10/10	5/8	0/10	
26	NT	7/10	2/10	0/10			20/20	5/10	0/10	
26	MES	10/10	7/10	0/10		2	10/10	7/10	1/10	
26	MMS	4/5	9/10	3/10			5/5	10/10	7/10	1/10
28	NT	20/20	6/10	0/10			4/10	0/10		
28	MES	10/10	9/10	0/10		6	7/10	0/10		
28	MMS	5/5	9/10	4/10			5/5	4/10		
49	NT	20/20	20/20	6/20			0/20			
49	MES	10/10	10/10	0/10		16	3/10	0/10		
49	MMS	8/8	9/9	8/8	2/8		7/8	4/10		
50	NT	20/20	15/20	2/20			6/20	0/10		
50	MES	7/10	7/10	0/10		18	9/10	0/10		
50	MMS	7/8	6/6	3/4	1/8		4/8	0/10		
51	NT	7/20	0/20				4/10	0/10		
51	MES	5/10	0/10			32	10/10	0/10		
51	MMS	8/8	1/10				5/5	6/9	0/10	
53	NT	20/20	0/20				0/20	0/10		
53	MES	5/10	0/10			39	0/10			
53	MMS	10/10	0/10				4/5	6/9	3/8	
40	NT	20/20	0/10				0/20			
40	MES	8/10	0/10			41	0/10			
40	MMS	5/10	3/8				3/8			

[a] UR Nos 4, 5 7, 37, 1, 27 52 38 17 24, 29, 31 11, 13, 14, 19, 21, 22, and 33 inactive at 1 Gm /Kg
[b] UR Nos 3 34, 35, and 36 are convulsant (see text)
[c] UR Nos 12, 15, and 23 stimulate, then depress at 1 Gm /Kg (see text)
[d] NT = Neurotoxicity (see text)
[e] MES = Supramaximal electroshock technique (see text)
[f] MMS = Maximal Metrazol seizure test (see text)

(TD_{50}, 100 mg /Kg , ED_{50} M E S , 14 mg /Kg) compare favorably with those of Swinyard, *et al* (6), (phenobarbital—TD_{50}, 60 mg /Kg , ED_{50} M E S , 20 mg./Kg , and diphenylhydantoin—TD_{50}, 90mg /Kg , ED_{50} M E S , 10 mg /Kg) While the urazole compounds were relatively ineffective against M E S in non-neurotoxic doses, good anti-M M S activity was demonstrated

The protective index against M M S was determined for the more active members of the series The compounds selected were UR-2, 10, 20, 25, and 49 The protective indexes of trimethadione and phenobarbital were also determined These data are presented in Table III. Our data for the oral ED_{50} values of trimethadione and phenobarbital are in general agreement with the subcutaneous ED_{50} values of 198 mg /Kg for trimethadione and 9 mg /-Kg for phenobarbital sodium, obtained by Goodman, *et al* (4) The ratio of ED_{50} M E S /ED_{50} M M S was determined for the above compounds and diphenylhydantoin. These data are presented in Table IV The relative selectivity of UR 2, 20, and 49 as M M S antagonists is particularly striking. Our data for the ratio of ED_{50} M. E S /ED_{50} M. M. S for phenobarbital sodium and trimethadione are in general agreement with Goodman, *et al* (4), and Chen, *et al* (9) Our ratio for diphenylhydantoin lies between the ratio of 0 19 obtained by Goodman, *et al*, and the ratio of 2 1 obtained by Chen, *et al* In the study of Goodman, *et al.* (4), the compounds were given subcutaneously, whereas we administered the compounds orally Chen, *et al* (9), used a modification of the intravenous Metrazol infusion technique, and an electroshock stimulus of different intensity than that used by us These differences may account for the discrepancies obtained in the three laboratories

Time-duration studies on UR 20, 100 mg /Kg (Fig. 1), indicate that this compound has a peak effect fifteen minutes after administration It possesses little, if any, protective action one hour after administration The neurotoxicity tests indicated that all active members of this series are rapidly absorbed from the intestinal tract and have a relatively short duration of action

UR 3 produced hyperactivity at a dose of 1 Gm./-Kg Only 3 out of 10 mice survived the electroshock stimulation, whereas we have found that control mice usually survive this test

Compounds UR 34, 35, and 36 produced con-

TABLE III.—TOXICITY, ANTI-M. M. S. POTENCY, AND PROTECTIVE INDEXES OF UR 2, 10, 20, 25, 49, TRIMETHADIONE, AND PHENOBARBITAL[a]

Drug	Time,[b] min	Neurotoxicity, TD_{50}, mg./Kg.	Potency, ED_{50}, mg./Kg.	Protective Index, P. I.
UR 2	30	295 (163–380)	70 (48–100)	4.2 (2.6–6.2)
UR 10	30	168 (125–221)	48 (34–60)	3.5 (2.5–5.3)
UR 20	30	452 (322–634)	73 (52–90)	6.6 (4.4–10.4)
UR 25	30	294 (224–385)	54 (37–70)	5.4 (3.8–7.9)
UR 49	30	151 (99–195)	38 (26–47)	4.2 (2.9–6.0)
Trimethadione	90	975 (760–1390)	236 (196–281)	4.0 (3.0–5.5)
Phenobarbital sodium	120	60 (47–77)	9 (6–12)	6.9 (4.8–10.1)

[a] Values in parentheses represent the 95% confidence interval.
[b] Time elapsed after administration of drug before Metrazol was injected.

TABLE IV.—ANTI-M. M. S. POTENCY, ANTI-M. E. S. POTENCY, AND RATIO ED_{50} M. E. S./ED_{50} M. M. S. FOR UR 2, 10, 20, 25, 49, TRIMETHADIONE, PHENOBARBITAL, AND DIPHENYLHYDANTOIN[a]

Drug	Time,[b] min.	M. M. S. Potency ED_{50}, mg./Kg.	M. E. S. Potency ED_{50}, mg./Kg.	Ratio ED_{50} M. E. S./ED_{50} M. M. S.
UR 2	30	70 (48–100)	508 (404–619)	7.4 (5.1–10.4)
UR 10	30	48 (34–60)	136 (100–174)	2.9 (1.7–3.3)
UR 20	30	73 (52–90)	438 (359–528)	6.0 (4.6–8.1)
UR 25	30	54 (37–70)	262 (111–355)	4.8 (3.3–7.2)
UR 49	30	38 (26–47)	201 (133–271)	5.6 (3.9–8.5)
Trimethadione	90	236 (196–281)	1082 (923–1262)	4.6 (3.8–5.7)
Phenobarbital sodium	120	9 (6–12)	21 (17–25)	2.4 (1.7–3.3)
Diphenylhydantoin	180	11 (9–12)	14 (11–17)	1.3 (1.1–1.7)

[a] Values in parentheses represent the 95% confidence interval.
[b] Time elapsed after administration of drug before stimulus was delivered.

vulsions at 1 Gm./Kg. The mice receiving compound 34 exhibited only clonic seizures and died of respiratory failure within one hour. A dose of 500 mg./Kg. produced clonus within ten minutes. Seven out of 10 animals died within two hours. Compound 35, 1 Gm./Kg., produced death in 6 out of 10 mice within thirty minutes, while the remaining animals showed mild clonus and tonic flexor seizures for an hour. At 500 mg./Kg., all had periodic mild clonus and appeared stimulated for approximately forty-five minutes. UR 36, 500 mg./Kg., produced clonus which rapidly changed to tonic flexor and tonic extensor seizures. Four out of five mice died within ten minutes after dosage. Neither UR 35 nor UR 36, at doses of 100 mg./Kg. and 300 mg./Kg. had any apparent effect on the sleeping time of mice receiving 100 mg./Kg. of hexobarbital administered i. p. (Table V).

Compounds UR 12, 15, and 23, 1 Gm./Kg., caused initial stimulation which was followed by profound depression. Table VI shows the mean sleeping time of UR 12 and UR 23, 300 mg./Kg., as compared to a similar dose of hexobarbital.

All active compounds were studied for their ability to protect against death produced by i. p. injections of 2.5 mg./Kg. of strychnine. Only UR 8, 1 Gm./Kg., provided significant protection and was comparable to 250 mg./Kg. of Flexin[4] and 500 mg./Kg. of mephenesin (Table VII).

DISCUSSION

The qualitative changes in biological activity induced by variations in the substituent groups on the urazole nucleus provokes many speculations as to the nature of these relationships. In the following paragraphs certain of these relationships are detailed. However, the possibility exists that these speculations are not very reliable. The compounds designated as inactive produced no observable effects during a period of ninety minutes following oral administration of a dose of 1 Gm./Kg. At least three possibilities are pertinent to the present discussion: (a) These derivatives are delayed in onset beyond the period of observation, (b) they are not absorbed following oral administration, or (c) they are unable to alter the tested phenomena. The rapid onset and short duration of action of the active urazoles studied seems to render (a) unlikely. These

[4] The Flexin was kindly supplied through the courtesy of Dr. Charles F. Kade, Jr., McNeil Laboratories, Inc.

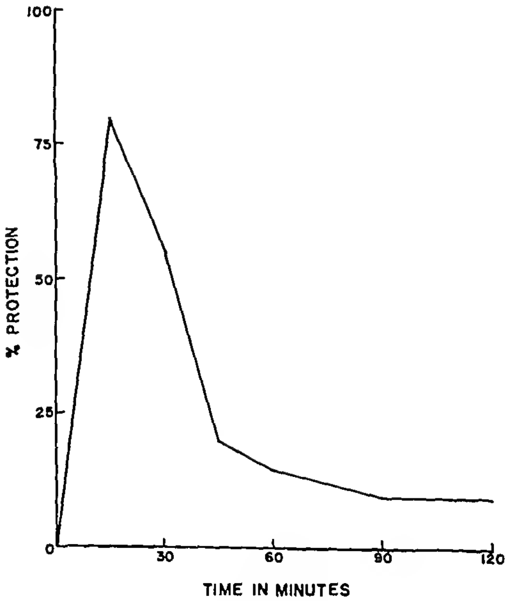


Fig 1 —Duration of action of UR 20, 100 mg /Kg.

TABLE V —EFFECT OF UR 35 AND UR 36 UPON THE MEAN SLEEPING TIME OF HEXOBARBITAL, 100 MG /KG I P IN THE MOUSE

Hexobarbital, 100 mg /Kg i p with	Mean Sleeping Time, min	95% Confidence Interval
Nothing	21	13 9–28 1
UR 35 100 mg /Kg (oral)	24	11 8–36 2
UR 35 300 mg /Kg (oral)	30	18 2–41 8
UR 36 100 mg /Kg (oral)	29	19 5–38 5
UR 36 300 mg./Kg (oral)	23	12 6–33.4

TABLE VI —MEAN SLEEPING TIME OF UR 12, UR 23, AND HEXOBARBITAL, 300 MG./KG , ORALLY

Drug	Mean Sleeping Time, min	95% Confidence Interval
UR 12	47	24 6– 69 9
UR 23	94	76 5–112 6
Hexobarbital	59	40 0– 77 8

TABLE VII.—ABILITY TO PREVENT DEATH FROM I. P. INJECTION OF 2 5 MG./KG. OF STRYCHNINE

Drug	Oral Dose	Time,[a] min	No of Animals Surviving/ No of Animals Tested
UR 8	1 Gm./Kg.	30	4/8
Flexin	250 mg./Kg	60	4/10
Mephenesin	500 mg./Kg.	5	4/10

[a] Time elapsed after administration of drug before strychnine was injected.

same considerations apply to (*b*) and, in addition, the presence of activity in closely related derivatives would seem additional evidence negating the validity of (*b*). We have therefore assumed that (*c*) is in fact true, and upon this hypothesis we have based the following discussion of structure-activity-relationships

The results obtained from this series indicates that at least one phenyl group on the adjacent nitrogen atoms (R_1 or R_2, Table 1) is essential for anticonvulsant activity. The urazole nucleus alone (UR 4) is devoid of anti-MES and anti-MMS activity. Note also UR 4, 7, and 37. Cyclohexyl substituents on both the R_1 and R_2 positions (UR 17) are also inactive, whereas a cyclohexyl substituent on one symmetric nitrogen with a phenyl group on the other (UR 20) was one of the more active compounds tested. Isopropyl substituents on both R_1 and R_2 (UR 34, 35, and 36) confer convulsant properties

If R_1 or R_2 is phenyl, all other substituents except hydrogen on the adjacent nitrogen atom can confer anticonvulsant activity. (Compare UR 1 and UR 3 with 8, 9, 10, 12, 23, 25, 28, 40, 20, 12, 2, and 49). It is interesting to note that UR 3 and UR 8 have similar structures except for a reversal of the methyl group from the R_3 position (UR 3) to one of the adjacent nitrogen atoms (UR 8) resulting in a change of activity from stimulatory (UR 3) to anticonvulsant (UR 8).

The R_3 substituent must be small or relatively polar for maximal anticonvulsant activity. A hydrogen atom on the R_3 position possesses the greatest activity. Substitution of a methyl group for the hydrogen atom results in a reduction in potency. Any group larger than a methyl group, unless it is somewhat polar, produces little, if any, anti-MES or anti-MMS activity. (Compare the series UR 8, 9, 46, and 47; UR 10 and 26; UR 12 and 17; UR 20, 42, 44, and 29; UR 15, 45, 43, 30, and 31; UR 2, 6, 16, 18, 39, 11, 13, 14, 19, 21, 22, 32, and 33; UR 49, 50, 53, 51, and 52). The only example in this series of a completely polar group (UR 41) was found to possess little activity.

It is difficult to formulate structure-activity-relationships for convulsant, antistrychnine, or hexobarbital-like activity in view of the few compounds possessing these actions.

The variety of actions noted in this series is certainly of interest, although the anti-MMS activity is perhaps the most striking property of the urazoles

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Gibberellic Acid: Toxicologic and Pharmacologic Studies*

By EUGENE T. KIMURA, PATRICK R. YOUNG, and KAZIMIR STANISZEWSKI

The plant growth stimulant, gibberellic acid, was tested for its toxicologic and pharmacologic properties in mice, rats, cats, guinea pigs, rabbits, and dogs. It was essentially nontoxic when tested by various routes of injection in mice for its acute toxicity. Both subacute toxicity in mice, and subchronic toxicity in dogs and rats showed the compound to be asymptomatic and free of pathologic changes, histologically. The following studies were likewise negative: circulation and respiration, barbiturate potentiation, antipyresis, anticonvulsant activity, intestinal motility, blood cholesterol, and blood glucose.

In 1932, Kurosawa (1) reported that culture filtrates of the fungus *Gibberella fujikuroi* produced a substance which caused an overelongation phenomenon of rice plants. Within the last several years, interest has centered on the practical application of this plant growth-promoting substance called the gibberellins or gibberellic acids in the fields of horticulture and agriculture. Though voluminous reports have appeared on the effects of this substance in plants, relatively few have appeared concerning its possible effects on mammalian organ systems. It was, therefore, considered of interest to report on our toxicologic and pharmacologic studies with this plant stimulant. The results obtained provide the basis for this report.

EXPERIMENTAL

Material.—The sodium salt of gibberellic acid[1] was used as an aqueous solution in concentrations up to 30% for intravenous toxicity studies. For mouse studies, the volume injected intravenously was kept below 0.5 ml. and the speed of injection at 0.5 ml./minute. Methylcellulose suspensions of the drug were used for oral toxicity studies. For subchronic toxicity studies in rats, aqueous solutions of the drug were freshly prepared every 3–4 days and kept under refrigeration when not in use. For all other parenteral studies, drug solutions were prepared on the day of the experiment.

Acute Toxicity in Mice.—The drug was tested for its acute oral, intravenous, and intraperitoneal toxicity in groups of adult male Rockland mice, weighing between 18–24 Gm., using 10 animals per dose level. Animals were observed for two weeks following medication.

Subacute Toxicity in Mice, Dogs, and Rats.—Ten adult Rockland mice were medicated subcutaneously for fourteen days at 1 Gm./Kg., and 5 mice were dosed intravenously for five days at 2 Gm./Kg. To minimize the possibility of irritation due to repeated trauma of subcutaneous injection, four different areas were selected and used in sequential order during medication. Saline controls were run concurrently. Animals not sacrificed for autopsy were observed for an additional nine days.

Two litter-mate dogs of both sexes were medicated orally via capsule with 1 Gm./Kg. of the drug, six days a week for a period of ninety days. Six male and six female Holtzman rats were treated orally with a solution of the drug via feeding tube at the same dose level for a similar period. The following laboratory procedures were performed: urinalysis (clinical and microscopic), complete blood counts (hemoglobin, red, white, and differential), function tests (liver and kidney), and blood chemistry (glucose, nonprotein nitrogen, and bilirubin).

Circulatory and Respiratory Studies.—Dogs and cats, anesthetized with Nembutal sodium, were used to record circulatory changes from the carotid. Intrathoracic and intrapulmonic fluctuations in dogs were recorded with tambours via Jackson-spike and tracheal cannula. Drug injections were made into the femoral vein.

C.N.S. Effects.—The effect of the gibberellins on barbiturate potentiation was tested in groups of 10 male albino mice, pretreated either intravenously or orally with the drug at dose levels of 500 and 1,000 mg./Kg., respectively. Following pretreatment periods of ten minutes for the intravenous route, and forty-five minutes for the oral route, the animals were injected intravenously with 30 mg./Kg. of Pentothal sodium.

Anticonvulsant activity was evaluated by the use of a battery of three tests as previously outlined by Toman, Everett, and Richards (2). It consisted of the Metrazol, "psychomotor" and supramaximal electroshock tests.

Antipyretic activity was measured by taking normal temperatures hourly over a three-hour period in groups of four female Holtzman rats, followed by an initial intramuscular injection of 1.5 ml. of 15% Brewer's yeast. Eighteen hours later, the animals were challenged intramuscularly with a 1 ml. pyrogenic booster injection of yeast suspension. At this point the animals were medicated orally with the gibberellins at dose levels of 150 and 500 mg./Kg. and its possible antipyretic effects tested at one-half hour intervals for a period of two and one-half hours.

In Vitro Intestinal Motility.—The effect of the drug against spontaneous intestinal motility was tested on the rabbit ileum, using the Magnus method. The effects of the drug against standard spasmogens and relaxants (acetylcholine, histamine, barium, pilocarpine, epinephrine, and atropine) were carried out with guinea pig and rabbit ileum.

* Received July 21, 1958, from Department of Pharmacology, Research Division, Abbott Laboratories, North Chicago, Ill.

[1] Gibberellic acid, Abbott (Peoria Strain, 91% X and 9% A).

Sixteen rabbits and four guinea pigs were used in these studies

Other Studies —Total blood plasma cholesterol determinations were made in groups of six mice given the gibberellic acid in their diet at a level of 0 17%, equivalent to about 249 mg /Kg daily for two weeks The method of Zak, *et al* (3), was used to determine total cholesterol

Blood glucose levels were determined four hours after oral doses of 250 mg /Kg and 500 mg /Kg of the gibberellins in groups of three rats/dose level

The effect of this plant growth stimulant on testicular growth in mammals was ascertained in groups of eight male Holtzman rats, weighing approximately 100 Gm The animals were medicated orally five days a week for a period of four and one half weeks with 1 Gm /Kg and with 0 5 Gm /Kg of the drug

Ten per cent aqueous solutions of the drug were applied daily to the backs of male mice, six days a week for three and one half weeks in order to see whether irritation or histologic changes could be induced

RESULTS

Intravenous acute toxicity studies in mice showed no deaths at doses as high as 4 Gm /Kg Orally, doses as high as 15 Gm /Kg were not lethal No symptoms were observed Intraperitoneally, a dose of 2 Gm /Kg was likewise asymptomatic There were no delayed deaths

Subacute toxicity studies, both intravenously for five days at 2 Gm /Kg and subcutaneously for fourteen days at 1 Gm /Kg, in mice yielded negative findings with respect to both organ toxicity and symptomatology Four mice on intravenous and four mice on subcutaneous medication were sacrificed for autopsy at the end of the previously described medication periods The following organs of the above eight animals were sectioned for histopathologic examination heart, lungs, spleen, liver, kidneys, adrenals, testes, pancreas, stomach, small and large intestines, and bone marrow None of the organs examined showed any changes which could be attributed to drug action Ninety day subacute toxicity studies in both dogs and rats at a dose level of 1 Gm /Kg showed no changes at autopsy and histological examination[2] which could be attributed to drug action Both male and female dogs, four male and four female rats from the experimental group as well as two male and two female rats from the control group were sacrificed at the end of the ninety day period, and the following organs sectioned for histopathologic examination heart, lungs, thymus, thyroid, aorta, liver, kidneys, spleen, adrenals, gonads (testes, ovary, tube, and uterus), stomach, small and large intestines, pancreas, brain, and bone marrow The animals gained weight satisfactorily and urinalysis, hematology, function tests, and blood chemistry failed to show any pathologic changes

During the ninety day experimental period, the female dog doubled her weight from 4 8 Kg to 9 7 Kg The male gained 8 5 Kg from his initial weight of 5 7 Kg to his terminal weight of 14 2 Kg During the medication period, the male consumed 781 Gm of the drug and the female, 648 Gm

An additional 10 male and 12 female rats were added to the 1 Gm /Kg dose and have now been on medication at this level for the past fourteen months These experiments are still in progress

Intravenous injections of the drug into three anesthetized dogs at dose levels up to 0 5 Gm /Kg did not affect either the circulatory or respiratory functions Intravenous injections of 100 mg /Kg into two anesthetized cats did not alter the blood pressure response to either serotonin or epinephrine

Pentothal sodium sleeping times in mice were not altered by gibberellic acid treatment Oral pretreatment with 1 Gm /Kg of the drug was likewise ineffective in altering the duration of sleep induced with 100 mg /Kg of hexobarbital sodium, intraperitoneally, in mice

The drug showed no anticonvulsant activity in mice at dose levels of 1, 2, and 4 Gm /Kg, orally (4) It likewise did not influence the temperature of either normal rats or animals in which fever had been induced with yeast

Studies on the effect of gibberellic acid on spontaneous motility of the isolated rabbit ileum showed the plant stimulant to have no effect at 1, 2 5, and 5 mg /ml bath concentration The highest concentration used was also without any effect on spasms induced in isolated guinea pig and rabbit ileum by barium (0 32 μg /ml), acetylcholine (0 05–0 5 μg /ml), and pilocarpine (0 25–2 0 μg /ml), and on relaxation induced with atropine (0 01 μg /ml) Results were likewise negative against histamine (0 01 μg /ml) in the guinea pig ileum and against epinephrine (0 01 μg /ml) in the rabbit

Blood cholesterol and blood glucose levels were essentially negative (4) Total blood plasma cholesterol values were 99 mg % for those animals medicated with gibberellic acid in their diet at a level of 0 17% as compared with 94 mg % for control animals, the average per cent blood glucose change, plus its standard deviation, for those animals given 250 and 500 mg /Kg of the drug were $-1\% \pm 14$ and $-6\% \pm 5$, respectively Each animal served as its own control Testicular growth, as measured by weight and histological examination, was not affected Daily application of the drug onto the skin of mice was also without any effect

These results, coupled with those of Peck, *et al* (5), indicate that gibberellic acid is a relatively nontoxic and pharmacologically inert compound when given orally, parenterally, topically, or when tested on isolated organ systems

SUMMARY

Gibberellic acid was tested by various routes in mice for its acute toxicity and found to be essentially nontoxic Both subacute toxicity in mice and subchronic toxicity in dogs and rats showed the compound to be asymptomatic and free of pathologic changes, histologically The highest observed nontoxic (and asymptomatic) doses in mice as determined by acute toxicity testing were as follows 4 Gm /Kg, intravenously, 15 Gm /

[2] Histopathologic examinations were carried out in the Pathology Section under the direction of Dr R J Stein

Kg., orally; and 2 Gm./Kg., intraperitoneally. Subacutely, the following doses were tolerated by mice, rats, and dogs for the times indicated: mice, 2 Gm./Kg., intravenously, for five days; mice, 1 Gm./Kg., subcutaneously, for fourteen days; and rats and dogs, 1 Gm./Kg., orally, for ninety days. The following studies were likewise negative: circulation and respiration, barbiturate potentiation, antipyresis, anticonvulsant activity, intestinal motility, blood cholesterol, and blood glucose.

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A Note on the Phytochemical Investigation of *Cecropia peltata* L.*

By NYDIA M. KING and NOELIA HADDOCK

A preliminary investigation of the leaves of *Cecropia peltata* L. has been made. Glycosidal material was obtained from the alcohol extract and also from the aqueous extract. No sterols or alkaloids were found.

Cecropia peltata, L. (*Ambaiba peltata*, Kuntze) commonly known as yagrumo (trumpet tree) is a tall tree with large lobed leaves which are dark green above and silvery-white beneath It is the type species of the many Cecropias native of the West Indies and continental tropical America (1).

Several therapeutic uses have been claimed for the various parts of the tree. The bark and buds are said to be astringent, the roots and bark have been used as an emenagogue, while the leaves have been employed in acute pulmonary infections, as an antiasthmatic, and as a sedative in nervous conditions such as chorea (2). Grosourdy (2) reports that when using a decoction of the leaves as a sedative the patient experienced a state of passiveness and slowness in all his muscular movements and his heart rate was slowed and made more rhythmical. No cumulative action or side effects, such as digestive tract disorders like those caused by the continuous use of digitalis, were observed. It was found to have a diuretic effect. In view of these observations, yagrumo was thought to be a possible substitute for digitalis (3).

A survey of the literature revealed that very little chemical and pharmacological investigations of *Cecropia peltata* L. have been reported. Valeri and Narvácz (4) showed that decoctions and infusions of 15 to 30 Gm. of the leaves per liter were nontoxic to guinea pigs when given by hypodermic injection. They found that the preparations had diuretic and laxative effects in guinea pigs, also causing transitory drowsiness. *In vitro* they had no inhibitory power over pathogenic bacteria of the respiratory apparatus.

Due to its potential use in medicine it seemed desirable to effect a thorough chemical investigation of *Cecropia peltata*, L. The first part of this work is reported here.

* Received June 28, 1958, from the College of Pharmacy. University of Puerto Rico, Rio Piedras

EXPERIMENTAL

The plant material used in this investigation consisted of the leaves collected locally, dried at 60°, and ground.

Preliminary examination tests, carried out as described by Rosenthaler (5), indicated the presence of tannins, mucins, proteins, and reducing substances while alkaloids and saponins were shown to be absent. According to the Stas-Otto method the material did not contain alkaloids or glycosides Further examination was made following the general method outlined by Rosenthaler (6).

Sixteen grams of the ground leaves was extracted successively with the solvents mentioned in the order given below. A Soxhlet apparatus was used for all the extractions, except for the extraction with water. The latter was done by maceration in a mechanical shaker and the solvent was removed under reduced pressure. The nonaqueous solvents were allowed to evaporate in air. The values of per cent extractives obtained were as follows: petroleum ether 1.16%, ethyl ether 1.93%, absolute alcohol 6 93%, acetone 3 86%, chloroform 0 84%, and cold water 6 75%

It was observed that the alcoholic extract deposited rosette-like crystals embedded in dark brown matter. A small amount of the crystalline deposit was readily soluble in water and the aqueous solution gave positive tests for carbohydrates and/or glycosides with a naphthol solution, and also for reducing sugars with Fehling's solution. The noncrystalline portion of the alcoholic extract gave a strong Fehling's test after it had been heated with dilute hydrochloric acid.

The aqueous extract gave positive tests for proteins, alkaloids, and reducing substances Upon clarification with a solution of lead acetate a residue was obtained which, after boiling with dilute hydrochloric acid, reduced Fehling's solution. Negative results were obtained when the rest of the extracts were tested for the usual substances which might be present.

Except for the deposit formed in the alcoholic extract, the different extracts did not yield any crystalline material, even after prolonged refrigeration.

Sixteen rabbits and four guinea pigs were used in these studies

Other Studies—Total blood plasma cholesterol determinations were made in groups of six mice given the gibberellic acid in their diet at a level of 0 17%, equivalent to about 249 mg /Kg daily for two weeks The method of Zak, *et al* (3), was used to determine total cholesterol

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A survey of the literature revealed that very little chemical and pharmacological investigations of *Cecropia peltata* L. have been reported. Valeri and Narváez (4) showed that decoctions and infusions of 15 to 30 Gm. of the leaves per liter were nontoxic to guinea pigs when given by hypodermic injection. They found that the preparations had diuretic and laxative effects in guinea pigs, also causing transitory drowsiness *In vitro* they had no inhibitory power over pathogenic bacteria of the respiratory apparatus.

Due to its potential use in medicine it seemed desirable to effect a thorough chemical investigation of *Cecropia peltata*, L. The first part of this work is reported here.

* Received June 28, 1958, from the College of Pharmacy, University of Puerto Rico, Rio Piedras

EXPERIMENTAL

The plant material used in this investigation consisted of the leaves collected locally, dried at 60°, and ground.

Preliminary examination tests, carried out as described by Rosenthaler (5), indicated the presence of tannins, mucins, proteins, and reducing substances while alkaloids and saponins were shown to be absent. According to the Stas-Otto method the material did not contain alkaloids or glycosides. Further examination was made following the general method outlined by Rosenthaler (6).

Sixteen grams of the ground leaves was extracted successively with the solvents mentioned in the order given below. A Soxhlet apparatus was used for all the extractions, except for the extraction with water. The latter was done by maceration in a mechanical shaker and the solvent was removed under reduced pressure. The nonaqueous solvents were allowed to evaporate in air. The values of per cent extractives obtained were as follows: petroleum ether 1.16%, ethyl ether 1.93%, absolute alcohol 6.93%, acetone 3 86%, chloroform 0.84%, and cold water 6.75%.

It was observed that the alcoholic extract deposited rosette-like crystals embedded in dark brown matter. A small amount of the crystalline deposit was readily soluble in water and the aqueous solution gave positive tests for carbohydrates and/or glycosides with a naphthol solution, and also for reducing sugars with Fehling's solution. The noncrystalline portion of the alcoholic extract gave a strong Fehling's test after it had been heated with dilute hydrochloric acid.

The aqueous extract gave positive tests for proteins, alkaloids, and reducing substances. Upon clarification with a solution of lead acetate a residue was obtained which, after boiling with dilute hydrochloric acid, reduced Fehling's solution. Negative results were obtained when the rest of the extracts were tested for the usual substances which might be present.

Except for the deposit formed in the alcoholic extract, the different extracts did not yield any crystalline material, even after prolonged refrigeration.

Twenty grams of the ground leaves was extracted with cold water and a 10% solution of lead acetate was used to clarify the extract A small amount of a colorless amorphous powder, m. p 202–205°,[1] was isolated from the lead acetate precipitate by following the usual procedure. Attempts to crystallize this material from acetone, methanol, and a mixture of ethanol and water were not successful, but the melting point changed to 214–215°. The material was insoluble in water, 5% hydrochloric acid, and ether; soluble in 5% sodium hydroxide, less soluble in 5% sodium bicarbonate; soluble in concentrated sulfuric acid with the production of a white precipitate upon dilution with water. It gave a positive Fehling's test after it was boiled with dilute hydrochloric acid.

Crystalline material was obtained from the basic lead acetate precipitate. This material gave a positive Fehling's test, but much more so after boiling with dilute hydrochloric acid. Due to the small amount available it was not possible to investigate it further at the time.

[1] Melting points are uncorrected

SUMMARY

1. The leaves of *Cecropia peltata*, L. were extracted with various solvents.

2. Impure crystalline material and an amorphous colorless powder, m. p 214–215°, both giving presumptive tests for glycosides, were isolated.

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A Note on a Simplified Technique for the Administration of Isotopes to Animals*

by RALPH M. LILIENFELD, CHARLES A. ROSS, and ROBERT W. CASE

Quick freezing of a tracer dose of a radioactive isotope provides a simple, precise method of administration to experimental animals and minimizes loss of radioactive substance.

IN ANIMAL ABSORPTION STUDIES using tracer doses, some error inevitably results from loss of the administered isotope which clings to syringe, catheter, and other instruments requisite to the administration. To minimize this loss, we have adopted the following technique: The measured amount of isotope is pipetted into 1/2 of a gelatin capsule. The capsule is carefully capped and dropped immediately into a test tube containing ethyl alcohol which is immersed in a freezing bath of dry ice and ethyl alcohol. The isotope freezes solid within the gelatin capsule in less than fifteen seconds with no loss of radioactive substance. Prior to its administration, the capsule is removed from the test tube with forceps and exposed to room temperature for a period of thirty seconds. The capsule dissolves within the stomach within five minutes after its oral administration and absorption of the isotope from the G.I. tract is not affected by the freezing process.

* Received July 30, 1958, from the Roswell Park Memorial Institute, Buffalo, N Y

A Note on a Method for Thymectomizing the Rat*

By ALFRED P. IACOBUCCI and HOWARD J. JENKINS

THE PROCEDURE for thymectomy in rats has been described in various texts and laboratory manuals. The descriptions of the procedures seem to be lacking in detail, however. It is the purpose of this paper to provide a more complete and detailed description of the surgical technique involved.

In the method reported, male, albino rats of the Carworth-Wistar strain, ranging in weight from thirty-five to fifty grams, are used. Rats of this size are more easily employed since older rats have developed more fatty tissue and heavier fascia. In addition, the thymus glands of smaller rats are larger and more easily removed.

* Received July 22, 1958, from the Edwin Avery Brewer and Howard D Brewer Pharmacology Laboratories of The Massachusetts College of Pharmacy, Boston
A procedure reported in a thesis submitted by Alfred P Iacobucci in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy

ANESTHESIA

Anesthesia of moderate depth should be employed. Ether is the ideal anesthetic; but it must be used with extreme caution, otherwise animals may be lost when the deeper planes of anesthesia are reached suddenly and without warning.

In the preliminary work, pentobarbital sodium was administered at the rate of 4 to 5 mg. per 100 Gm. of body weight. Induction with this agent was rapid, but the duration of anesthesia was sufficient to complicate the state of postoperative shock that was encountered. For this reason pentobarbital sodium was considered an unsatisfactory agent for this method.

In other animals, a combination of pentobarbital sodium (3 mg per 100 Gm of body weight) and Evipal sodium (6 mg per 100 Gm. of body weight) administered intraperitoneally was tried. This

TABLE I.—THE EFFECT OF SURGERY TIME AND DURATION OF ANESTHESIA ON MORTALITY RATE[a]

Time, min.: Gland Removal/ Completed Operation	Recovery Time, min (Pentobarb)	Mortality, %	Recovery Time, min. (Pentobarb. and Evipal)	Mortality, %	Recovery Time min. (Ether)	Mortality, %
5/10	120 plus	60	60–90	50	15–30	20
2–5/10	90–120	50	45–90	50	10–15	10
2–5/5–10	90–120	40	45–90	30	10–15	10
2/5	90–120	20	45–90	20	10–15	5

[a] In the above table the time required for the removal of the thymus gland is correlated with the elapsed time of the entire surgical procedure, including closure of the chest cavity, while the recovery time represents the duration of the period between the onset of anesthesia and the regaining of consciousness.

anesthetic mixture shortened the recovery period, and thereby diminished to some extent the possibility of death from postoperative shock complicated by respiratory failure. Even so, some difficulties were evident with this mixture, since Evipal is not well tolerated by young rats.

After considerable experience with its use, ether was selected as the anesthetic for the method. Though some danger of respiratory infection attends its use, recovery is rapid and generally satisfactory.

SURGICAL PROCEDURE

When the rat has been anesthetized, it is placed on its back on an operating board, head toward the operator. The legs are tied to the board and a bit passed behind the incisor teeth and secured in position to hold the head stationary. Hair is removed from the region of the chest and throat. An incision about 2 cm. in length is made along the midline of the thoracic wall from the level of the third rib to a point just beyond the anterior end of the sternum. Here a pointed scissors is inserted under the anterior end and the sternum is cut down to the level of the second rib. (This incision should not exceed 1 cm. in length.) It is essential that the operator work rapidly from this point. Accordingly, the first loop of a continuous suture is started prior to the removal of the gland, so that the chest wall can be closed quickly following extirpation. A size "O," type B, noncapillary thread of silk or nylon attached to a three-quarter inch, half-round, atraumatic, gastrointestinal needle is employed. With blunt probes, the underlying tissue is pushed aside and the chest cavity entered at a point just above the branching of the trachea. The anterior end of the gland is thus exposed. At this point, some respiratory difficulty is exhibited by the rat as a result of the loss of negative pressure in the chest cavity. Both lobes of the gland are held with blunt forceps and a steady even pull is exerted while the gland is gently freed from its surrounding connective tissue. Care is taken to avoid penetrating the great vessels of the heart. When the gland is completely removed, all negative pressure in the chest cavity is lost. Thus, prompt closure of the chest wall is imperative. (It may be necessary to close the incision with a hemostat while suturing.) The cut edges of the thoracic wall are brought into approximation with suture and the skin closed with wound clips. While recovering, the animals exhibit Cheyne-Stokes respiration, but the mortality rate should not exceed 5%. The closed incision is painted with a suitable antiseptic to diminish the possibility of bacterial infection. Postoperative shock is not severe, and the animals begin to gain weight on the day following surgery.

Four or five minutes are required for the entire operation. The actual removal of the gland and closing of the chest cavity should be accomplished within a two-minute period if the mortality rate is to be kept low.

Communications to the Editor

2-(*o*-Tolyl)-cyclohexanol: A Possible Cardioplegic Agent

Sir:

We wish to report that 2-(*o*-tolyl)-cyclohexanol was found capable of suppressing myocardial contraction to the exclusion of significant effect on myocardial electrical properties. During exploratory pharmacological investigations with 2-(*o*-tolyl)-cyclohexanol one of us (G. H. B.) observed that this compound produced reversible mechanical cardiac arrest when perfused as a saturated aqueous solution through isolated cat hearts. The preliminary investigation has been extended to include observations on the isolated rabbit atrium and on the result of intravenous injection in the anesthetized dog. In the isolated preparation, 3 mg. per 100 ml. nutrient solution resulted in virtually complete inhibition of contraction within one minute. With complete mechanical asystole, the transmembrane atrial action potential was essentially unchanged, the membrane resting potential was not altered, and absolute refractory period was not changed. Threshold of electrical excitability increased approximately 10 per cent and stabilized. The

effect on contraction was slowly reversible by washing. In the intact dog, the injection of 60 mg./Kg. (i.v.) resulted in a rapid hypotensive effect leading to complete absence of arterial pressure within one minute. This was presumed to be due in part to depressed ventricular contraction, although it is not known to what extent the drug affects the vasculature directly. In the experiment described, the dog was pharmacologically sympathectomized. No change in spontaneous heart rate, PR interval, QRS duration, or ST duration occurred until many minutes after the onset of the hypotensive effect. Then complete AV block ensued and ventricular conduction slowed. This possibly was due to hypoxia resulting from cardiovascular syncope. Respiration was little affected until agonal gasping occurred.

2-(*o*-Tolyl)-cyclohexanol was prepared from *o*-methylphenyllithium and cyclohexene oxide by the method described by McKusick for the synthesis of the *meta* isomer (1). The product was fractionated through a Todd precise fractionation assembly with a 90-cm. Monel spiral column; b. p. 103–104° at about 1 mm.

Anal.—Calcd. for $C_{13}H_{18}O$: C, 82.07; H, 9.52. Found: C, 81.84; H, 9.27.

The I. R. spectrum of the pure liquid has an intense band at 3,320 cm.$^{-1}$ indicative of hydrogen-bonded hydroxyl groups and a less intense band at 3,450 cm.$^{-1}$ attributed to unassociated hydroxyl groups. The spectrum also has a sharp band at 964 cm.$^{-1}$, a region which falls within the range of characteristic absorption for cyclohexane derivatives (2). The spectra of nine substituted 2-arylcyclohexanols determined at this laboratory (3) all show a band within the range of 962–974 cm.$^{-1}$. The spectra were obtained on a Perkin-Elmer Model 21 spectrophotometer with NaCl prism.

The synthetic method used is expected to give the trans-isomer. This is substantiated by the fact that Cook, Hewett, and Lawrence (4) obtained only one isomer of 2-phenylcyclohexanol from phenyllithium and cyclohexene oxide under similar conditions and that this isomer is now known to be the trans-2-phenylcyclohexanol (5, 6). Investigation of the stereochemistry and of the stereospecificity on the pharmacological properties observed of this and other related compounds is in progress.

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ALAIN C. HUITRIC, THEODORE C. WEST, and ROBERT A. DURBIN
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GORDON H. BRYAN
School of Pharmacy, Montana State University, Missoula

Labiatae Family

Sir:

Chemical ontogenetic and phylogenetic considerations of the *Labiatae*, relating the occurrence of sterols and certain terpenes, include data (1) on two species of the *Umbelliferae*. Although conclusions were not established concerning the data derived by a perfunctory examination of the unsaponifiable fraction of extracts of these plants, the unqualified presence of the data is misleading. The reader's perception of chemical phylogeny is further inclined by the exclusion of *Marrubium vulgare* L. from the *Labiatae*.

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ARTHUR E. SCHWARTING
University of Connecticut, Storrs

Book Notices

The Interference Microscope in Biological Research By ARTHUR J HALE The Williams & Wilkins Co, Baltimore, exclusive U S agents, 1958 xi + 114 pp 14 x 22 cm Price $5

This is an instructive textbook designed to acquaint the biologist with the principles and techniques of interference microscopy An appendix discusses wave form and polarization of light, and gives examples of calculations The interference microscope, or microscopic interferometer, is particularly useful in the study of the processes involved in cellular metabolism

Ciba Foundation Symposium on the Neurological Basis of Behaviour Edited by G E W WOLSTENHOLME and CECILIA M O'CONNOR Little, Brown and Co, Boston, 1958 xi + 400 pp 13 5 x 20 5 cm Price $9

This book presents the papers and discussions of the symposium on the interrelations of neural and behavioral mechanism linking psychology with neurophysiology The subjects covered range from microphysiology of neuroses to phenomena of behavior and psychology Analytical, biological, neurophysical, pharmacological, biochemical, clinical, and strictly behavioral methods are considered A subject index is appended.

Symposium on Protein Structure Edited by ALBERT NEUBERGER John Wiley & Sons, Inc, New York, 1958 351 pp 15 5 x 23 5 cm Price $7 75

This book is a record of the papers and discussions at the symposium on protein structure sponsored by the Protein Commission of the Section of Biological Chemistry of the International Union of Pure and Applied Chemistry held at Paris, July 1957 The subjects are grouped under the headings General problems and methods, Specific proteins, Proteolytic enzymes, Ribonuclease, Tobacco mosaic virus, Other proteins and peptides A subject index is appended

Water and Electrolyte Metabolism in Relation to Age and Sex Ciba Foundation Colloquia on Ageing, Vol 4 Edited by G E W WOLSTENHOLME and MAEVE O'CONNOR Little, Brown and Co, Boston, 1958 xii + 327 pp 13 5 x 20 5 cm Price $8 50

This compilation of the proceedings of the fourth colloquium on ageing covers many problems, including genetic control of electrolyte metabolism in the erythrocytes, glandular secretion of electrolytes, hormonal aspects of water and electrolyte metabolism in relation to age and sex, the development of acid-base control, severe magnesium deficiency, and the ability of the organism to maintain normal cellular states under various nutritional conditions, the role of the kidney in electrolyte and water regulation in the aged, and age and renal disease A subject index is appended

Phosphorus and Its Compounds—In Two Volumes Vol I Chemistry By JOHN R VAN WAZER Interscience Publishers, Inc, New York, 1958 xiii + 954 pp 15 x 23 cm Price $27 50

This is a comprehensive text on phosphorus and its compounds, which is presented with the purpose of laying a foundation for phosphorus chemistry as a new, separate discipline of chemistry The text is divided under the chapter headings The phosphorus atom, its nucleus and electronic structure, Interaction between atoms, with especial reference to phosphorus chemistry, Systematic chemistry of phosphorus and its compounds, Elemental phosphorus and the metal phosphides, Hydrides, halides, and pseudohalides of phosphorus and their organic derivatives, Oxides, sulfides, nitrides, and related compounds of phosphorus, Lower oxyacids of phosphorus, their salts and esters, Structure and properties of the condensed phosphates, Orthophosphoric acid, its salts and esters, Individual chain phosphates (pyro, tripoly, tetrapoly-, and pentapoly-phosphates as well as Kurrol's and Maddrell's salts), Ring and branched phosphates, Amorphous phosphates, including phosphate glasses, condensed phosphoric acids, and phosphate esters, and Halo-, peroxy-, thio-, and amidoacids of phosphorus, their salts, esters, and related compounds The text material contains many diagrammatic formulas, illustrations, and tabulated data, and many references are given Appendixes give a table of 187 phosphate minerals, a collection of single bond energies and distances with electronegativity differences for use in calculations concerning phosphorus compounds, and thermodynamic data on phosphorus compounds Author and subject indexes are included

Encyclopedia of Chemistry (Supplement) Edited by GEORGE L CLARK and G G HAWLEY Reinhold Publishing Corp, New York, 1958 vi + 330 pp 17 5 x 25 5 cm Price $10

This *Supplement*, including more than 200 additional reviews, is stated to be the only volume that will be added to the first volume The arrangement of the text is the same as in the original volume which was cited in THIS JOURNAL, 46, 454(1957) The concise discussions in the *Supplement* include Sedatives and tranquilizers, Sunproofing agents, Drugs (legal aspects of marketing), Antihistamines, Antimalarials, and Dentifrices

Physico Chimie Biologique et Médicale By CHRISTAIN BÉNÉZECH Masson et Cie, Paris, 1958 vii + 684 pp 17 x 24 cm Price 8,000 fr

This textbook (in French) is intended for biochemists, physiologists, and others in the related fields of medicine The text is divided into four main parts under the headings Matter and its transformations, Homogeneous states of living matter (gas liquid-solution), Dispersed systems and macromolecules, and Elements of physico chemical biology A subject index is appended

Radioactive Isotopes in Clinical Practice. By EDITH H. QUIMBY, SERGEI FEITELBERG, and SOLOMON SILVER. Lea & Febiger, Philadephia, 1958. 451 pp. 15.5 x 23.5 cm. Price $10.

This book describes radioisotopes, tells how they act, and explains their clinical uses in diagnosis and treatment of disease. The text is divided into the three separately authored parts: Basic physics, Instrumentation and laboratory methods, and Clinical applications. The three authors have worked as a team in this field, and their views correlate the information for physicians and technicians. The hazards of radiation and the procedures required to avoid them are stressed Author and subject indexes are appended. Pharmacists in radioisotope centers will find this a useful reference.

Initiation Mathématique a la Physique Médicale et a la Biologie. By JULIEN GUELFI Masson et Cie., Paris, 1958. viii + 220 pp. 16.5 x 24 cm. Price 4,000 fr.

This book (in French) is intended as a mathematics text for students of medicine and pharmacy in particular. It ranges from algebraic calculations, elementary geometry, and trigonometry, through calculations of probabilities and statistics.

Disinfection and Sterilization. By G. SYKES. D. Van Nostrand Co., Princeton, 1958. xviii + 396 pp. 15.5 x 25 cm. Price $10.75.

This book, incorporating recent applications and discussions on theory and mode of action of disinfection and methods of sterilization, should be useful to medical bacteriologists, pharmacists, and industrial bacteriologists. The text is divided into six major parts. Part I discusses theory of disinfection and methods of testing disinfectants and antiseptics. Part II covers methods of sterilization. Part III is devoted to air disinfection and sterilization. Part IV discusses disinfection of viruses. Compounds used as disinfectants and antiseptics are taken up in Part V. Part VI deals with methods of preservation in relation to foods, pharmaceutical products, and industrial materials. Author and subject indexes are appended.

Accepted Dental Remedies 1959. 24th ed. American Dental Association, Chicago, 1958. xiv + 210 pp. 15.5 x 20.5 cm. Paperbound. Price $3.

This book contains expanded sections on dental therapeutics. The monographs have been reviewed by the Council and its consultants and have been rewritten in the light of current scientific information. The reorganized format, which made last year's edition so popular, has been retained with minor additional improvements. A discussion of the prescription use of fluoride preparations is included in the chapter on Fluoride Compounds.

As in earlier editions, Accepted Dental Remedies 1959 includes information concerning drugs of recognized value in dentistry, drugs of uncertain status more recently proposed for use by the dentist, and some drugs once employed extensively but now generally regarded as obsolete. Only brands of drugs which the Council has classified in Group A are included in the book.

Guide Pratique de la Laborantine. 3rd ed. By J. MARLIANGEAS. Vigot Freres, Editeurs, Paris, 1958. 144 pp. 16 x 24 cm. Price 1,000 fr.

This book (in French) is intended for beginning laboratory technicians and medical students. It describes techniques and procedures for bacteriologic, hematologic and serologic, histologic, and chemical methods used in the clinical laboratory.

Biochemical Engineering. Edited by R. STEEL. The Macmillan Co., New York, 1958. 328 pp. 14 x 21.5 cm. Price $7.50.

This book is based upon postgraduate lectures on the industrial application of biochemical processes, particularly as they apply to unit processes in fermentation. The text is divided under the chapter headings: Principles of biochemical engineering; Scope of biochemical engineering; Microorganisms and their activities; Substrates for fermentation processes; Sterilization of equipment, Air and media; Development of anaerobic fermentation processes: Acetone-butanol; Oxygen supply and demand in aerobic fermentations; Development of aerobic fermentation processes: Penicillin; Equipment design; Recovery of fermentation products; and Present trends and future developments. A subject index is appended.

Organic Syntheses. Vol. 38. Edited by JOHN C. SHEEHAN. John Wiley & Sons, Inc., New York, 1958. vii + 120 pp. 15 x 23 cm. Price $4.

Methods of synthesis are provided in this volume for the compounds in the list which follows: 2-Amino - 4 - anilino - 6 - (chloromethyl) - s - triazine; 2 - Benzylaminopyridine; Benzyltrimethylammonium ethoxide; 4-Bromo-2-heptene; 2-Bromo-3-methylbenzoic acid; 3-(*o*-Chloroanilino) propionitrile; Dibromaocetonitrile; Dicyclopropyl ketone; Diethyl methylenemalonate; 5,5-Dimethyl-2-*n*-pentyltetrahydrofuran; Diphenylacetaldehyde; N- Ethyl- *p*- chloroanilinine; 5- Formyl - 4 - phenanthroic acid; Hendecanedioic acid; 3-Hydroxytetrahydrofuran; 6-Ketohendecanedioic acid; 2-Methyl-2,5-decanediol; *trans*-2-Methyl-2-dodecenoic acid; 2-Methylenedodecanoic acid; β-Methylglutaric anhydride; Methyl Hydrogen Hendecanedioate; 1-Methylisoquinoline; Methyl *p*-tolyl sulfone; Monobenzalpentaerythritol; Monobromopentaerythritol; Monovinylacetylene; 1-Nitroöctane; 1,4 - Pentadiene; α - Phthalimido - *o* - toluic acid; *trans*-Stilbene oxide; and 2-Vinylthiophene.

Treatment in Internal Medicine. By HAROLD T. HYMAN. J B Lippincott Co., Philadelphia, 1958. xiv + 609 pp. 17.5 x 25.5 cm. Price $12.50.

A compendium of current treatment procedures designed to serve as a ready and reliable source of information for the busy physician. Its pharmaceutical interest lies in the discussion and evaluation of many drugs.

Physiology of Spinal Anesthesia. By NICHOLAS M. GREENE. The Williams & Wilkins Co., Baltimore, 1958. xi + 195 pp. 15 x 23 cm. Price $6.

A documented review and evaluation of the physiology of spinal anesthesia.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Cinnoline Chemistry IV*

Infrared Spectra

By RAYMOND N. CASTLE, DAVID B. COX†, and JOHN F. SUTTLE‡

The infrared spectra of eighteen cinnolines have been determined. A consistent absorption band at approximately 6.35 microns has been observed and this has been assigned to the interaction effects of the ring double bonds of the cinnoline ring, namely the —C=C—, the —N=N— and the —C=N— bonds. This absorption band appears to be characteristic of the cinnoline ring system.

THE SYNTHESIS of compounds containing the cinnoline ring system has been a subject of interest in this laboratory for some time (1, 2, 3). The compounds described in this paper were prepared for pharmacological screening. In the course of the preparation of the compounds described (3) it became desirable to study the infrared spectra of a number of cinnolines.

Attempts to hydrolyze α-4-cinnolyldiphenylacetonitrile to the corresponding carboxylic acid which was then expected to decarboxylate to α-4-cinnolyldiphenylmethane gave back a quantitative yield of the starting material. Analytical data indicated the presence of the nitrile group. This was confirmed by a weak but definite absorption band at 4.5 microns indicative of the —C≡N stretching vibration (Table I). These data confirm the presence of the nitrile group. Presumably this particular nitrile group is too hindered for hydrolysis to take place, even when heated sixty hours in 60 per cent sulfuric acid solution.

The infrared spectra of 18 cinnolines have been determined. In all the cinnolines studied an absorption band occurs in the 6.35 micron region. Examination of the data in Table I under the heading Cinnoline Ring will disclose absorptions varying from 6.20 microns to 6.40 microns with the majority falling between 6.34 and 6.38 microns. It is expected that interactions of the other groups present in the compounds would shift the absorption bands somewhat. This absorption band has been assigned, on the basis of the 18 compounds studied, to the interaction effects of the ring double bonds. The overall absorption at this wavelength is probably due to the combined interaction effects of —N=N—, —N=C— and —C=C— bonds. The absorption spectrum of liquid pyridazine (4) shows a very strong absorption band at 6.38 microns (1567 cm.$^{-1}$).

The spectrum of 4-hydroxycinnoline fails to reveal on OH stretching vibration in the 3 micron region, however, a shoulder is found at 6.17 microns which is interpreted to indicate a ring, conjugated carbonyl. These data suggest that 4-hydroxycinnoline exists in the tautomeric 4-cinnolone structure, II, in the solid state. This is in agreement with the work of Hearn, Morton, and Simpson (5) in which 4-hydroxycinnoline was described as approximately a 70-30 tautomeric mixture of forms I and II with the cinnolone structure predominating in alco-

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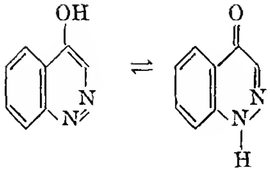
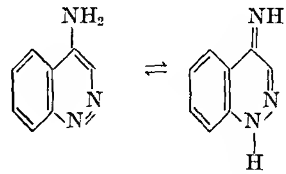


I II

solution. The spectral evidence for the structure of 6,7-dimethoxy-4-hydroxycinnoline is in agreement with the 4-cinnolone structure. There is no apparent OH stretching vibration

The spectrum of 4-aminocinnoline reveals weak absorption band at 3.02 microns. In contrast a very strong absorption band is noted at 5.97 microns which is indicative of the —C=NH grouping. Three other absorption bands at wavelengths that can be attributed to NH bending vibrations are noted at 8.97 (m), 12 23 (m), and 13.05 (m). A fourth at 12.94 (s) can be attributed to N—H deformation vibration. Based on this evidence, 4-aminocinnoline, III should exist predominantly in the imino structure, IV, in the solid state. This interpretation is in agreement with the conclusions reached by Hearn, Morton, and Simpson (5) based upon ultraviolet absorption spectra.



III IV

The absorption band due to the nitrile group in α-4-cinnolyldiphenylacetonitrile is shifted from 4.65 microns to 4.50 microns in this strongly hindered nitrile. The intensity of this absorption is weaker than any other nitrile studied. Four strong intensity (13.17, 13.38, 14.21, and 14.32)

Table I —Infrared Spectra of Cinnolines

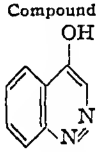
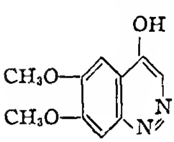
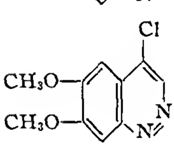
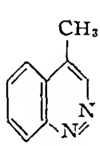
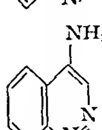
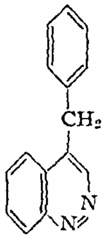
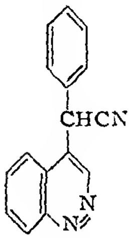
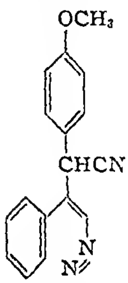
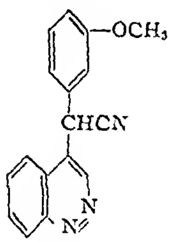
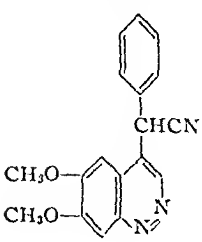
Compound	Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group	Miscellaneous
	Wave Length in Microns				
		6 40 sh			6 17 sh alpha, beta-unsaturated aminoketone 9 27 m
		6 30 ? sh 6 33 ? w	7 80 s 7 88 s	6 17 m 9 24 m 8 13 s 8 23 s 8 52 m 8 93 m	 11 55 m 12 02 m 12 20 m 13 75
		6 20 ? w		7 72 s 7 95 m 8 04 m 8 20 m 8 34 m 8 63 m 8 81 m	6 53 6 69 9 77 w 9 95 w 10 43 m 11 76 m 12 10 m 12 57 m C—Cl
		6 37 w			6 67 w methyl deformation 8 68 w methyl rocking vibration 12 22 w methyl rocking vibration 12 82 w 13 22 14 25 w
		6 38 s			3 02 w NH stretching 5 97 s C=NH 6 63 s 7 70 s 7 93 s 8 23 s 8 97 m NH—bending 9 26 m 9 85 m 11 65 m 12 23 m NH bending 12 94 m NH deformation 13 05 s NH bending

TABLE I (*Continued*)

Compound	Wave Length in Microns Cyano Group	 Cinnoline Ring	 Phenyl Ring	 Methoxy Group	 Miscellaneous
		6 35 m	6 69 m		12 95 m 13 21 s 13 97 s 14 30 m } out of plane C—H deformation vibration
	4 64 s	6 34 s	6 20 w 6 59 s		3 12 s 8 12 m 8 80 m 9 18 m 13 00 m 13 15 m 13 40 m 14 25 s } out of plane C—H deformation vibration
	4 64 m	6 35 m	6 25 m 6 68 m	7 80 s 7 95 s 8 06 m 8 52 m 8 67 w 8 95 w	9 85 m 12 03 m 12 40 w 13 00 m } out of plane C—H deformation vibration
	4 65 s	6 37 s	6 20 w 6 30 m 6 63 s	7 85 m 8 04 m 8 29 m 8 81 m	12 70 w 12 95 m 13 45 w 14 25 m } out of plane C—H deformation vibration
	4 68 w	6 40 w	6 19 m	7 72 s 7 95 m 8 05 s 8 19 s 8 34 s 8 63 s 8 82 s	9 77 w 9 95 m 10 42 m 11 75 m 12 10 m 12 57 m } out of plane C—H deformation vibration

and two medium intensity (14.05 and 14.70) absorption bands are found in the spectrum of this hindered nitrile. These are attributed to the out of plane C—H deformation vibrations to be expected in a molecule of this type.

EXPERIMENTAL

The infrared spectra were determined from pastes in Nujol with a Perkin Elmer Model 21 double beam spectrophotometer

The compounds were prepared by methods previously described (1, 2, 3) except for 4-aminocinnoline

4-Aminocinnoline.—Three grams of 4-chlorocinnoline was heated for eight hours at 95° in a capped "coke" bottle with 150 ml of 28% aqueous ammonia. The reaction mixture was evaporated under reduced

TABLE I (*Continued*)

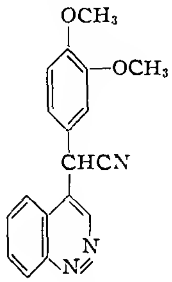
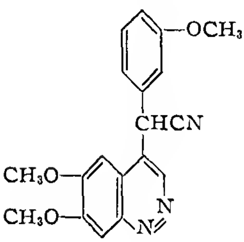
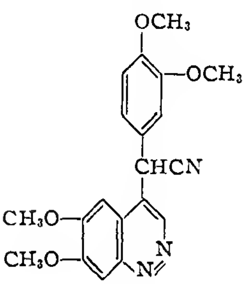
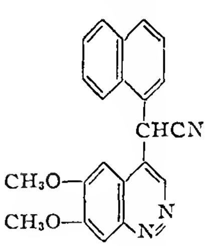
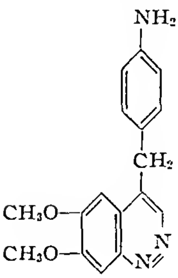
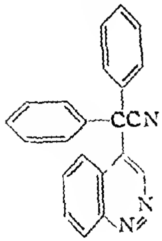
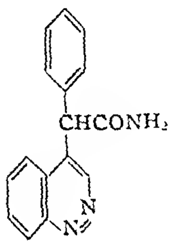
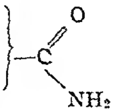
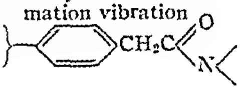
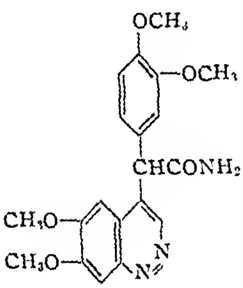
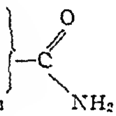
Compound	Wave Length in Microns Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group	Miscellaneous
	4 65 w	6 35 m	6 25 w 6 70 m	7 80 w 7 94 m 8 07 m 8 28 m 8 52 m 8 62 m 8 81 m	11 40 w 11 70 m 12 46 m out of plane C—H deformation vibration 13 00 m 13 35 m o-disubstituted phenyl
	4 65 m	6 38 s	6 16 m 6 25 w 6 68 s	7 87 m 8 00 m 8 16 m 8 27 m 8 35 m 8 97 m 9 20 m	11 52 m 11 93 m 12 05 w 12 32 w 12 55 m 13 15 w 14 25 w out of plane C—H deformation vibration -mono substituted phenyl
	4 64 s	6 36 s	6 15 m 6 25 m 6 63 s	7 92 s 8 05 s 8 14 s 8 28 s 8 56 s 8 76 s 8 95 s 9 15 s 9 48 m	9 78 s 10 10 s 11 58 w 11 90 s 12 00 m 12 25 s 13 08 m 14 20 w out of plane C—H deformation vibration o-disubstituted phenyl
	4 65 s	6 33 s	6 15 s 6 55 m (naphthyl)	7 63 m 7 87 s 7 95 m 8 08 m 8 25 s 8 58 m 8 82 m 9 24 m	9 52 s 9 85 m 9 98 s 11 39 m 11 82 m 12 08 m 12 45 m 12 80 s 13 38 m out of plane C—H deformation vibration naphthyl
		6 35 w	6 50 w 6 60 m	7 65 m 7 90 m 8 10 m 8 27 m 8 57 m	6 21 m aniline (or phenyl ?) 9 65 m 9 75 m 10 12 m 11 37 w 11 88 m 12 00 m 12 87 w out of plane C—H deformation vibration
	4 50 w	6 38 m	6 20 w 6 30 w 6 72 s		7 14 m 8 49 m 8 70 m 8 75 m 9 34 m 9 70 m 9 99 m

TABLE I (*Continued*)

Compound	Wave Length in Microns				
	Cyano Group	Cinnoline Ring	Phenyl Ring	Methoxy Group	Miscellaneous
					11 03 w 11 22 w 12.20 w 12 40 w 12 60 w out of plane C—H deformation vibration 13 17 s 13 38 s 14 05 m 14 21 s 14 32 s 14 70 m
		6 38 m	6 70 w		3 03 s 3 18 s 5 95 s  6 13 s 7 10 m 7.70 w 8 03 w 8 86 w 8 80 m 10 48 w 11 88 w out of plane C—H deformation vibration 12 30 w 13 25 s  14 23 s 14 93 m
		6 30 m	6 70 s	7 78 m 7 90 m 8 05 m 8 15 s 8.29 s 8 55 m 8 72 s	3 03 s 3 18 s 5 97 s  6 18 s 7 15 m 9 63 m 9 76 s 10 13 m 10 35 w 11 38 w 11 80 s out of plane C—H deformation vibration 12 00 m 12 45 m 12 68 w 13 12 m o-substituted phenyl 14 85 m

pressure A yield of 2 76 Gm (97%) of crude 4-aminocinnoline melting at 208–212° was obtained After purification by solution in dilute hydrochloric acid, precipitation with dilute sodium hydroxide, and subsequent crystallization from aqueous ethanol (charcoal), there was obtained 2 1 Gm (87%), of fine yellow crystals m p 215 5–218° (Keneford, Schofield, and Simpson (6) report 212–213°) The identity of the 4-aminocinnoline, prepared in this manner, was confirmed by the preparation of 4-acetylaminocinnoline Seven-tenths of a gram of 4-aminocinnoline was refluxed for six and one-half hours with a mixture of 3 ml of acetic anhydride and 8 ml of dry pyridine The amine was slowly soluble in the acetic anhydride-pyridine mixture. A part of the pyridine-acetic anhydride mixture was removed by vacuum evaporation and the residue was poured into cold water The product which separated was purified by crystallization from ethanol (charcoal). The purified product amounted to 0 22 Gm of fine crystals melting at 277° with decomposition

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Studies on the Pharmacology of Cobalt Chloride*

By H. R. MURDOCK, Jr.

The studies reported show that at dosage levels approximating those used in therapeutics, cobalt is absorbed in the rat in significant quantity. Oral administration has been shown to produce demonstrable increases in erythropoietin in the blood and, as a result, peripheral blood values respond to an extent proportional to the dosage administered.

COBALT THERAPY is widely used in the treatment of certain forms of anemia, both in Europe and in the United States. However, its mechanism of action remained obscure prior to the demonstration by the Jacobson group (1) that its erythropoietic effect was due to increased erythropoietin formation.

Earlier pharmacologic studies dealing with absorption, excretion, toxicity, and metabolism of cobalt were based largely on parenteral administration. Since, however, oral use represents the principal therapeutic application, it was considered important to study these same factors upon oral administration and to relate these findings to previous work.

Our studies with oral cobalt administration form the basis of this report.

MATERIALS AND METHODS

Absorption and Excretion.—Radioactive cobalt chloride solution was given orally in single individual doses by metal stomach tube to fasted adult albino rats. The solutions were prepared with sufficient Co^{58} chloride so that each dose represented approximately 1 μc of radioactivity, they were administered at dosage levels of 0.25 mg /Kg , 1 25 mg /Kg , 5 mg /Kg , and 10 mg /Kg. of elemental cobalt The animals were immediately placed in metabolism cages and the pooled urine and feces collected separately from each group during a seventy-two-hour period Three to six animals comprised each dosage level group.

The radioactivity was determined at intervals in aliquot portions of both urine and feces in a well-type scintillation counter Feces were digested with concentrated hydrochloric acid prior to counting

Determination of Erythropoietin.—We have modified the method of Goldwasser, *et al* (1), to permit the oral administration of cobalt Large fasted rats were given 100 mg./Kg. of elemental cobalt, as cobalt chloride, orally by metal stomach tube They were exsanguinated twenty hours later, and the plasma obtained by centrifuging the heparinized blood. Two milliliters of this plasma was injected intraperitoneally into fasted rats weighing 120 to 150 Gm for two successive days. On the third day, 1 to 2 μc of Fe^{59} were injected into the external jugular On the next succeeding day, blood was taken from the heart and the radioactivity counted in a well-type scintillation counter. The Fe^{59} utilization was calculated and compared with controls Hemoglobin was also determined at the same time and rats with abnormal values were discarded.

Hemopoietic Studies.—Thirty adult male rats, 225 to 250 Gm , were given 10 mg /Kg of elemental cobalt, as cobalt chloride, orally by metal stomach tube five days a week A similar group of twenty-six rats received an equal volume of isotonic saline by metal stomach tube. All animals were maintained throughout the experiment on a standard diet and were kept in cages subject to identical conditions of temperature, etc

The erythrocyte count, hemoglobin, and hematocrit determinations were done at frequent intervals during the one hundred fifty-day period of study. At the end of the study, all rats were autopsied and tissues taken for microscopic pathologic examination.

Blood for hematologic determinations was collected in heparinized vials from the tail of lightly etherized animals Erythrocyte determinations were done by standard methods using a Spencer "Bright-Line" counting chamber. Hemoglobin was determined by the cyanmethemoglobin method. Hematocrit determinations were done using Van Allen tubes Blood volume determinations were not considered necessary because of the well-established fact that cobalt administration has no significant effect on blood volume (2)

Toxicity.—The oral LD_{50} was determined by the usual methods in adult fasted rats Ten rats were used at each of the six dosage levels

In determining chronic toxicity, one group of eight rats weighing approximately 225 Gm received 10 mg /Kg of elemental cobalt orally, as cobalt chloride, five days a week by metal stomach tube for two hundred forty days A second group of ten animals, weighing approximately 98 Gm , received 4 mg /Kg of elemental cobalt in the same manner for two hundred ten days At the conclusion of the test period, all animals were sacrificed, organs examined and weighed, and sections made for microscopic examination

RESULTS

Absorption and Excretion.—Absorption was rapid as evidenced by the appearance of labeled cobalt within thirty minutes in the urine of all animals tested (Table I) The principal portion of the absorbed isotope was excreted in the urine during the first few hours after administration

Of the total dose administered, approximately 27, 25, 15, and 12% was excreted in the urine at the dosage levels of 0 25, 1 25, 5, and 10 mg /Kg. of elemental cobalt, respectively Since biliary excretion is also known to be a factor, these values probably represent minimum absorption figures

Urinary excretion plotted against time on semilogarithmic paper appears to approach a straight-line relationship (Fig 1) Cobalt, therefore, adminis-

* Received September 26, 1958, from the Department of Biological Sciences, Lloyd Brothers, Inc , Cincinnati, Ohio
Technical assistance involved in these studies was given by E W. Tryczynski and J M Wagner Appreciation is extended to Dr L J Klotz for assistance in preparing this manuscript.

TABLE I.—URINARY EXCRETION OF DIFFERENT DOSAGES OF Co^{58} AFTER ORAL ADMINISTRATION OF LABELED COBALT CHLORIDE TO RATS

Hours After Admin.	0.25 mg. Co/Kg., %	1.25 mg. Co/Kg., %	5 mg. Co/Kg., %	10 mg. Co/Kg., %
0.5	1.1	1.2	.	
2.0	11.2	8.7	4.4	0.2
4.0	16.6	14.0	8.0	2.3
6.0	18.3	16.8	9.3	4.5
24.0	23.9	21.5	10.4	7.0
48.0	24.7	23.6	13.1	10.8
72.0	26.73	25.0	14.5	11.5

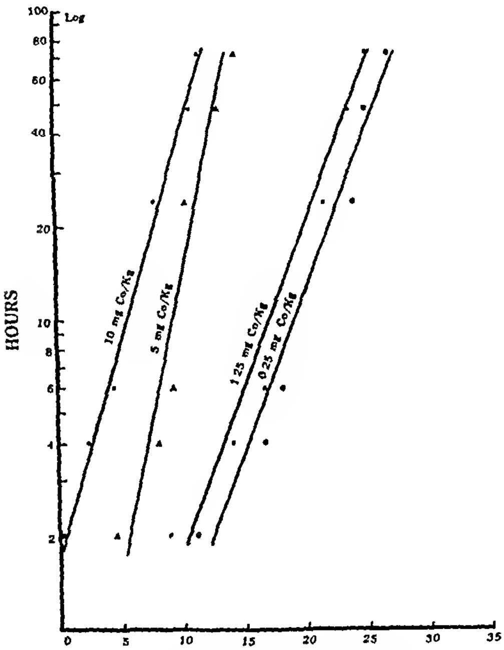

Fig. 1.—Urinary excretion of orally administered radiocobalt in rats. (Ordinate given in log-scale).

tered orally as the chloride, is absorbed in quantities roughly proportional to the dose given and is excreted principally in the urine.

Erythropoietin Determination.—Parenteral cobalt administration results in increased radioiron red cell incorporation due to erythropoietin formation (1). As an assay procedure, however, the method requires doses of cobalt near the LD_{50} level and mortality is high among the test animals.

Values obtained with orally administered cobalt at a dosage of 100 mg./Kg. were essentially similar to those found after parenteral administration, but the mortality was greatly decreased. There was no apparent inconsistency between results obtained by the two methods.

These findings, therefore, indicate both that oral cobalt is effective in enhancing the formation of erythropoietin and that the assay technique may advantageously be modified to use oral administration. Results are shown in Table II.

Hemopoietic Response.—Results are shown in Fig. 2. The values represent the average of each group of animals. It is of interest to note that a

TABLE II.—Fe^{59} RED CELL INCORPORATION IN RATS TREATED WITH COBALT-ENHANCED ERYTHROPOIETIN-RICH PLASMA

Group	No. of Rats	% Fe^{59} Incorporation	S.D.
Controls	32	8.3	±3.2
Erythropoietin-rich plasma (from oral cobalt treatment)	29	19.3	±4.9
Erythropoietin-rich plasma (from subcutaneous cobalt treatment)	5	21.3	

steady increase in the hematopoietic indexes occurred during the first sixty days and thereafter the polycythemia was maintained without further significant change. Since the blood indexes were essentially unchanged after ninety days, some of the rats were dropped from the experiment, leaving 16 rats in each group. Eleven animals in the control group and 12 cobalt-treated animals were carried throughout the experiment. Nine deaths occurred, mortality being due either to injury by the metal stomach tube or to anesthesia.

Table III shows the calculated mean corpuscular hemoglobin concentration and mean cell volume. It is evident that no significant changes occurred in these indexes and that, therefore, both cell size and hemoglobin content per cell, under cobalt therapy, remained approximately the same as in the normal animals.

These data are in keeping with the absorption data presented and indicate that oral cobalt is effective as a hemopoietic agent.

Toxicity.—The LD_{50} of cobalt chloride was found to be 144 mg. elemental cobalt per kilogram (Table IV). Toxic symptoms at the higher dosages appeared immediately after administration, and within six hours the rats either died or began to recover.

TABLE III.—HEMATOLOGIC INDEXES FOR COBALT-TREATED RATS

Days Treatment	Treated: M.C.H.C., %	Treated: M.C.V.	Controls: M.C.H.C., %	Controls: M.C.V.
0	36	54.6 cu. μ^a	34	56.0 cu. μ^a
30	37	54.0 cu. μ	35	55.3 cu. μ
60	38	53.6 cu. μ	39	52.5 cu. μ
90	37	53.4 cu. μ	39	
150	38	52.9 cu. μ	37	50.5 cu. μ

[a] Cu. μ—cubic microns, M.C.H.C.—mean corpuscular hemoglobin concentration, M.C.V.—mean cell volume.

TABLE IV.—LD_{50} DATA FOR COBALT CHLORIDE ADMINISTERED ORALLY TO FASTED RATS

Mg. Co/Kg.	No. Deaths / No. Injected
98	1/10
122	2/10
137	3/10
150	5/10
153	7/10
191	10/10

LD_{50} = 144(127–162) mg. Co/Kg.[a]

[a] Method of Litchfield and Wilcoxon (3).

TABLE V.—BODY WEIGHTS AND RATIO OF ORGAN TO BODY WEIGHTS OF RATS RECEIVING ORAL COBALT CHLORIDE

Group	Daily Dose mg. Co/Kg.	Duration of Expt., Days	Body Weight, Gm. Initial	Final	Ratio of Organ Weight, % Adrenal × 100	Kidney	Liver	Spleen
Control	.. (5)[a]	240	228	424	0 98	0.69	3.5	..
Cobalt chloride	10 (6)	240	224	382	0.98	0.75	3.8	..
Control	.. (7)	218	98	404	0.99	0 58	2.9	0.14
Cobalt chloride	4 (6)	218	98	396	1.00	0.67	3 4	0.20

[a] Number of animals in parenthesis

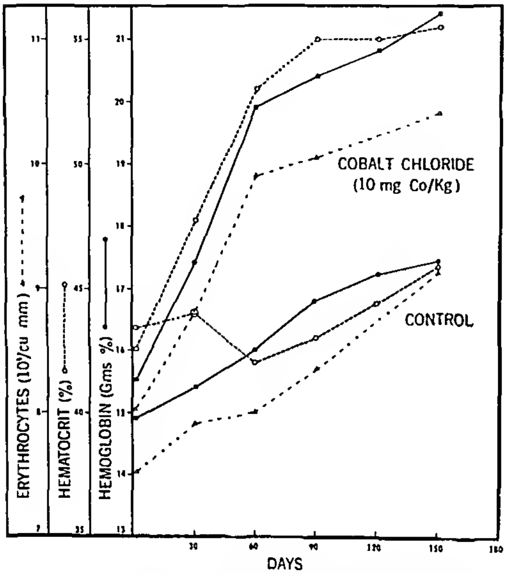

Fig. 2.—Average hemoglobin, hematocrit, and erythrocyte count of normal rats receiving 10 mg./Kg. elemental cobalt five days a week as compared with control animals.

Table V shows the initial and final body weights and ratio of organ weight to body weight of the rats receiving oral cobalt chronically. A few of the animals were lost due to injury caused by the daily stomach tubing It was considered that there were no significant differences in the body weights of controls and the animals receiving cobalt. The adrenal weights were approximately the same but the average weights of kidneys, livers, and spleens of the cobalt-treated groups were slightly heavier than the controls. Extra-medullary erythropoiesis, a common phenomenon in rats, may be responsible for the increased weights of spleen and liver.

Microscopic examination of the kidneys of the rats on the 10 mg./Kg. dosage level, but not on the 4 mg. dose level, revealed some necrosis of the cells lining the tubules. This was not unexpected since the kidney is the main pathway for the excretion of cobalt (4). The phenomenon is reversible, however, since examination of the kidneys of rats autopsied thirty days after cobalt administration was discontinued showed no necrosis and were normal compared to kidneys from control rats. Similar changes have been described by others (5).

The bone marrow of the rats receiving cobalt was hypercellular, particularly in the cells of the erythroid series. The Malpighian corpuscles of the spleen were enlarged, as described by Hopps, *et al.* (6), in rats receiving subcutaneous injections of cobalt chloride. Both of these changes are in accord with the polycythemia produced in these rats.

DISCUSSION

Studies on the extent of cobalt absorption, after oral administration, have been contradictory. Administration in animal feed has failed to provide definitive limits of dosage and there has been no attempt to correlate findings with clinically used dosage levels.

Minute doses are said to be completely absorbed (7) and larger dosages half absorbed (8), but others have reported insignificant gastrointestinal absorption (9). Therapeutically, adult dosage levels approximate 0.25 mg./Kg. of elemental cobalt, as cobalt chloride, per day. At this level, in our studies, absorption approximated 25% of the dose administered. Urinary excretion of the absorbed cobalt was relatively complete within twenty-four hours. These data are in accord with the findings of others (10).

Absorption after oral administration is sufficiently great and consistent to permit the demonstration of increased erythropoietin formation by cobalt. The consequence is a marked parallel increase in hemoglobin, red cell count, and hematocrit while the red cell indexes remain nearly constant and do not differ appreciably from those of controls Continued administration eventually results in a plateau for hemoglobin and hematocrit values, with the polycythemia being maintained at the new level. The response is proportional to the dosage used.

Acute toxicity studies indicate that cobalt chloride, given orally, is not very toxic and long-term administration indicates that at dosage levels approximating 10 times those used in therapy, no significant toxic effects are present. As has previously been reported (5), massive experimental doses may cause transient kidney damage.

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A Pharmacologic Evaluation of Certain Cobalt-Containing Hemopoietic Agents*

By H. R. MURDOCK, Jr., and L. J. KLOTZ*

Gastrointestinal absorption of cobalt-containing compounds appears to depend, in part, on a mechanism which concerns cobalt alone and is not dependent on the remainder of the molecule. The systemic toxicity appears to increase directly with the dissociation to supply cobalt ion. Cobalt disodium ethylenediamine tetra-acetate shows little dissociation and little systemic toxicity. The enhancement of erythropoietin formation can be accomplished in approximately the same degree by equivalent cobalt doses given as ionic cobalt, complex-ion, or difficultly dissociated chelated cobalt. This is subsequently reflected in peripheral blood indexes. Both cobalt polyglucopyranose and cobalt disodium ethylenediamine tetra-acetate have higher therapeutic indexes, as determined in the rat, than does cobalt chloride. Both would appear suitable for clinical trial. No evidence of chronic toxicity was found from prolonged oral administration of cobalt polyglucopyranose or cobalt disodium ethylenediamine tetra-acetate in the rat. Cobalt disodium ethylenediamine tetra-acetate (Copoietin A) appears to represent the most satisfactory type of cobalt compound tested and should be worthy of clinical trial as a therapeutic agent.

Although cobaltous chloride has received wide acceptance as a therapeutic agent in the treatment of anemia, its usefulness may be limited by gastrointestinal intolerance which often appears above dosage levels of about 100 mg. per day in humans. Furthermore, the "heavy metal effect" and the local irritant action of cobalt chloride practically preclude the use of effective doses by parenteral administration.

Numerous other cobalt compounds, usually of the "complex" type, have been described in the literature as having therapeutic merit because of decreased toxicity, decreased side effects, or practicality for parenteral use. None has found general medical acceptance at this time.

It is the purpose of this report to describe some of the pharmacologic properties of certain cobalt compounds which appear to offer advantages for possible therapeutic use. Three compounds studied were: cobalt-niacinamide complex, cobalt polyglucopyranose, and cobalt disodium ethylenediamine tetra-acetate.

MATERIALS AND METHODS

Preparation of Cobalt Compounds.—Cobalt-niacinamide complex was prepared according to published methods (1) It is a red crystalline material, soluble in water and alcohol, and contains about 15% elemental cobalt.

Cobalt polyglucopyranose is a complex prepared in this laboratory as follows. Five grams of "soluble starch" and 1 Gm. of cobaltous chloride in distilled water is brought to approximately pH 11 by the addition of 10% aqueous sodium hydroxide The formation of the complex is indicated by the appearance of a deep blue color. Excess isopropanol (one to three volumes) is then added. The complex precipitates and is washed free of alkaline chloride and any unreacted cobaltous chloride by washing with several portions of isopropyl alcohol. It appears that the cobalt atom is linked to the 1,4 oxygen bridge of the polyglucopyranose structure. It is greenish blue, noncrystalline solid, slightly soluble in water, insoluble in alcohol, and containing about 7% elemental cobalt.

The cobalt disodium ethylenediamine tetra-acetate chelate was prepared according to the method described by Schwarzenbach (2). It is a red crystalline material containing about 15% elemental cobalt which forms stable solutions in water and alcohol. Cobalt chloride used in certain of these studies was the commercial C. P. cobalt chloride hexahydrate with the purity verified by chemical assay.

Toxicity Studies.—Adult albino rats were used for the LD_{50} determinations. They were fasted overnight and the compound given the next morning so that the animals could be closely observed during the day. Ten rats were used at each dosage and at least five dosage levels were used for each compound.

Chronic toxicity studies were conducted on both weanling and adult rats. The cobalt compounds were administered orally five days a week by metal stomach tube. At the end of the study the rats were autopsied and the tissues and organs examined for gross as well as microscopic changes. Two groups of rats kept under identical conditions were given cobalt chloride and saline, respectively, as controls.

Absorption and Excretion Studies.—Labeled radiocobalt (Co^{58}) compounds were specially prepared for these studies. The method of preparation of the compounds used was that described above except that proper amounts of radiocobalt (Co^{58}) were used along with nonradioactive cobalt in the synthesis. The radioactive compound was given orally to fasted rats by metal stomach tube in a dosage representing 0.25 and 1.25 mg./Kg. of elemental cobalt. The lower dose closely approximates the common daily adult clinical dose of 60 mg. of cobalt chloride calculated on a weight basis. The rats were immediately placed in metabolism cages and the urine and

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Technical assistance involved in these studies was given by Joseph M Wagner, Emil W. Tryczynski, and Joseph B Vaughan

feces collected separately. Aliquot portions of the urine and of the acid-digested feces were counted in a well-type scintillation counter and the total amount of excreted cobalt was calculated.

Determination of Erythropoietin.—The procedure consisted of giving the cobalt compound orally to large eighteen-hour fasted rats at a dosage representing 100 mg./Kg. of elemental cobalt. Eighteen to twenty hours later the animals were exsanguinated under light ether anesthesia. Two milliliters of plasma from this blood was injected intraperitoneally into eighteen-hour fasted rats which were deprived of food throughout the assay. Twenty-four hours later, these animals received another 2-ml. injection of plasma. On the third day they were injected intravenously with 1 to 2 μc. of radioiron (Fe^{59}). Twenty-four hours later, blood was obtained from the heart, the Fe^{59} activity determined, and the percentage of erythrocyte incorporation of radioiron calculated. Details of the method are published elsewhere (3).

Hemopoietic Studies.—The methods used were similar to those reported previously (4). The compounds were administered five days a week to adult male rats by metal stomach tube. Hematologic data were determined at intervals. Blood samples were taken from the tail vein observing the usual precautions to avoid error. Erythrocyte counts were carried out using a Spencer "Bright-line" counting chamber; hemoglobin was determined photocolorimetrically; and hematocrit by the Van Allen method.

Urinary Calcium.—Because of certain theoretical considerations, it was thought desirable to determine the effect of one of our compounds (cobalt disodium ethylenediamine tetra-acetate) on urinary calcium excretion. For this purpose, total urinary calcium was determined by a modification of the method of Shohl and Pedley (5) in five human subjects. The values obtained on each subject during a control period of four days were taken as normal. This was followed by a four-day period during which time each subject took 440 mg. a day of cobalt disodium ethylenediamine tetra-acetate in divided doses. This dose represents somewhat more than four times the elemental cobalt dosage usually given as cobalt chloride. Diet was not restricted except that the subjects attempted to eat and drink roughly the same amount of high calcium foods each day they participated in the study.

RESULTS

Acute Toxicity.—The acute LD_{50} for cobalt chloride and for each of the three cobalt compounds, cobalt-niacinamide, cobalt polyglucopyranose, and cobalt disodium ethylenediamine tetra-acetate in the rat are given in Table I. Data are given in mg./Kg. of elemental cobalt represented by the dosage of the compound administered. Cobalt-niacinamide is of the same order of acute toxicity as cobalt chloride and in this respect does not appear to offer any advantages over the latter.

The intraperitoneal LD_{50} of cobalt polyglucopyranose represents 26 mg./Kg. of elemental cobalt. This is less than one-third as toxic as cobalt chloride. The onset of toxic symptoms at the higher dosages of cobalt polyglucopyranose appeared much later and were less severe than the symptoms following cobalt chloride injections. The rats that died following cobalt chloride injection usually did so within six hours; following cobalt polyglucopyranose, the majority of deaths occurred between twelve and twenty-four hours.

TABLE I.—ACUTE LD_{50} OF COBALT COMPOUNDS IN THE RAT

	LD_{50} in mg./Kg. Elemental Cobalt	
Compound	*Intraperitoneal*	*Oral*
Cobalt chloride	7 9 (7.4–8.5)[a]	144 (127–162)[a]
Cobalt-niacinamide	.....	134 (121–149)[a]
Cobalt polyglucopyranose	26.0 (1 96–35 1)[a]	>328
Cobalt disodium ethylenediamine tetra-acetate	>800	>1000

[a] Method of Litchfield and Wilcoxon (6).

It was not possible to obtain an exact oral LD_{50} with cobalt polyglucopyranose in the rat. Neither deaths nor serious toxic symptoms occurred at dosages representing less than 328 mg./Kg. of elemental cobalt. At this dosage, no immediate toxic symptoms were seen except for a black diarrhea appearing on the day following administration. However, two of the ten rats died on the third day. Autopsy of a rat which appeared quite ill after receiving this dosage revealed no abnormality other than hyperemia of the gastrointestinal tract. It was impractical to give higher dosages because of the relatively low solubility of the compound.

It was not possible to obtain either an oral or intraperitoneal LD_{50} in the rat with cobalt disodium ethylenediamine tetra-acetate. Orally, dosages representing as high as 1,000 mg./Kg. of elemental cobalt produced only mild toxic symptoms as did intraperitoneal injections containing up to 800 mg./Kg. of elemental cobalt. All the rats seemed to be fully recovered after twenty-four hours and none died. It was impractical to give higher dosages because of the large volume of even highly concentrated solution which was involved.

In comparison, the oral LD_{50} of cobalt chloride represents 144 mg./Kg. of elemental cobalt. The toxic symptoms appeared early and death usually occurred within six hours.

Rabbits injected intravenously with 25 mg./Kg. elemental cobalt as cobalt chloride invariably went into deep shock. The blood pressure fall was so severe that no bleeding took place at the site of injection.

Rabbits receiving twice this dose of elemental cobalt intravenously, given as cobalt disodium ethylenediamine tetra-acetate, showed no detectable toxic symptoms. Four times this dose (approximately 100 mg./Kg. elemental cobalt), administered as rapidly as possible, still produced no toxic symptoms except for a questionable increase in respiratory rate. There were no signs of shock and the rabbits remained alert and active.

Chronic Toxicity.—The results obtained from prolonged oral administration of the three cobalt compounds in the rat are shown in Table II. Data for the number of rats in each group refer to the number of rats autopsied at the conclusion of the experiment

TABLE II.—AVERAGE BODY AND ORGAN WEIGHT OF RATS RECEIVING COBALT COMPOUNDS ORALLY

Compound Administered	Dose mg./Kg., Co	No. Days on Expt.	Initial Body Wt., Gm.	Final Body Wt., Gm.	Ratio of Organ Wt. to Body Wt. %: Spleen	Adrenal × 100	Kidney	Liver
Cobalt PGP[a]	4 (8)[b]	240	92	356	0.15	1.0	0.55	3.1
Cobalt PGP	8 (8)	240	93	385	0.13	1.1	0.54	2.9
Cobalt EDTA[c]	4 (8)	180	98	366	0.22	1.2	0.66	3.5
Cobalt EDTA	8 (8)	180	98	398	0.18	1.0	0.66	3.1
Cobalt chloride	4 (6)	218	98	396	0.20	1.0	0.67	3.4
Saline	(7)	218	98	404	0.14	0.96	0.62	2.9
Cobalt EDTA	10 (8)	240	239	501		0.98	0.66	3.2
Cobalt EDTA	20 (6)	240	194	424		0.95	0.71	3.4
Cobalt EDTA	40 (6)	240	233	436		0.92	0.73	3.7
Cobalt chloride	10 (6)	240	224	382		0.98	0.75	3.8
Saline	(5)	240	228	464		0.98	0.68	3.5

[a] Cobalt polyglucopyranose. [b] Number of animals autopsied. [c] Cobalt disodium ethylenediamine tetra acetate.

since an occasional animal died due to injury caused by use of the metal stomach tube.

Administration of cobalt polyglucopyranose in a dosage representing 4 and 8 mg./Kg. of elemental cobalt to adult rats for two hundred forty days had no effect on the weight gain. At autopsy, no gross or microscopic pathologic findings were present. The ratio of the spleen, liver, and adrenal weights to the body weight were similar to the values obtained for the other groups. Values obtained for the kidney-body weight ratios were somewhat lower but the range overlapped values obtained with rats receiving cobalt chloride.

Cobalt disodium ethylenediamine tetra acetate in a dosage of 4 and 8 mg./Kg. of elemental cobalt had no effect on the weight gain of weanling rats. The ratio of the organ to body weights were within normal range and no gross or microscopic pathologic findings were present at autopsy.

Ten, 20, and 40 mg./Kg. of elemental cobalt given as cobalt disodium ethylenediamine tetra acetate also had no effect on the weight gain of adult rats when administered for two hundred forty days. At autopsy there were no gross or microscopic pathologic findings which could be attributed to the chelate. The organ to body weight ratios were also within normal range.

In contrast, continued oral administration of 40 mg./Kg. of cobalt as cobalt chloride causes severe anorexia and other toxic symptoms.

It is of interest to note that microscopic examination of the kidneys of those rats receiving 10 mg./Kg. of cobalt, as cobalt chloride, showed evidence of degeneration of the cells lining the tubules. This had previously been reported (7).

It was concluded that neither cobalt disodium ethylenediamine tetra-acetate nor cobalt polyglucopyranose showed evidence of toxicity when administered orally in the above dosages.

It has been shown that about 15 to 35% of the cobalt administered parenterally as cobalt disodium ethylenediamine tetra acetate is excreted as ionic cobalt (8). It follows, therefore, that a portion of the dose given probably frees a small amount of disodium ethylenediamine tetra acetic acid in the body. Previous work (9) has shown that this substance may chelate with and thus remove calcium from the body and that, in large intravenous doses, calcium deprivation and even tetany may result.

The usual human oral dosage level of about 400 mg. of cobalt disodium ethylenediamine tetra-acetate per day would provide 340 mg. of the disodium chelating agent, assuming complete dissociation of the cobalt chelate. This quantity could remove a maximum of about 40 mg. of calcium from the body per day.

Calcium excretion values for human subjects before and during daily oral administration of 440 mg. of cobalt disodium ethylenediamine tetra acetate are given in Fig. 1. The twenty-four hour urinary calcium varied greatly from subject to subject. One subject, E. T., with a past history of kidney stones, showed high values. The other subjects had no history of kidney or urinary pathology.

It is evident that there was no significant difference in the total calcium excretion during the experimental period from that during the control period and that administration of cobalt disodium ethylene diamine tetra acetate does not significantly promote the excretion of calcium in the doses used.

Absorption and Excretion.—Since the lack of systemic toxicity of cobalt disodium ethylenediamine tetra acetate and cobalt polyglucopyranose could be due to decreased absorption, it seemed desirable to determine whether the cobalt from these compounds was actually absorbed. Gastrointestinal absorption was estimated by preparing the two compounds with labeled radiocobalt and determining the urinary excretion of the radiocobalt following oral administration.

It is recognized that this is not a quantitative measure of absorption since cobalt is also excreted in the bile. The technique does, however, furnish evidence of absorption and provides useful data concerning the relative amounts absorbed.

Single oral doses of labeled radiocobalt chloride, cobalt polyglucopyranose, and cobalt disodium ethylenediamine tetra-acetate were given to represent 0.25 mg./Kg. or 1.25 mg./Kg. of elemental cobalt in each instance. Results are shown in Table III.

There appears to be little difference in excretion, and therefore probably in absorption, among the three compounds at the lower dosage level. Some decreases in absorption, however, appear to be present at the higher dosage. With both cobalt disodium ethylenediamine tetra acetate and cobalt polyglucopyranose, radiocobalt was found in the urine within half an hour, thus indicating rapid absorption. Over 95% of the cobalt excreted in the urine was recovered within twenty-four hours, indicating rapid absorption and excretion. When the urinary excretion values were plotted against time on semilog paper, a straight line was obtained. This is

TABLE III.—URINARY EXCRETION OF Co^{58} AS PER CENT OF ORAL DOSE

Hrs. After Dose	0.25 mg./Kg. Co, % CoEDTA[a]	CoPGP[b]	$CoCl_2$	1.25 mg./Kg. Co, % CoEDTA	CoPGP	$CoCl_2$
0.5	0.9	0.1	1.1	0.1	0.3	1.2
2.5	12.9	11.3	14.1	4.4	2.8	13.2
4.5	16.0	18.8	16.6	8.8	8.6	14.0
6.5	18.9	22.6	18.3	9.7	9.6	16.8
24.0	26.9	24.4	24.8	15.9	12.3	21.5
48.0	28.2	25.9		16.9	12.8	
72.0	28.3	27.0		17.3	13.1	
96.0	28.3	27.1	26.7	17.5	13.2	25.0

[a] Cobalt disodium ethylenediamine tetra acetate. [b] Cobalt polyglucopyranose.

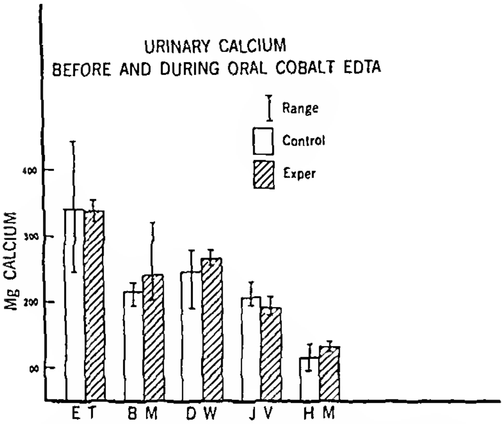

Fig. 1.—Urinary calcium excretion in human subjects before and during oral administration of cobalt disodium ethylenediamine tetra acetate. Each bar represents the average for four days.

taken as indicative of little or no retention of cobalt in the body.

Erythropoietin Production.—Since cobalt is absorbed when given in the form of these compounds, its physiologic availability from a nonionizing chelate such as cobalt disodium ethylenediamine tetraacetate was of interest. As a measure of availability of the cobalt component, the effect on the production of erythropoietin, the erythropoietic hormone, was determined.

Results are given in Table IV. They indicate that erythropoietin production was increased to approximately the same degree following cobalt disodium ethylenediamine tetra acetate, cobalt polyglucopyranose, and cobalt chloride administration.

Hemopoietic Effect.—To determine whether these erythropoietin increases would be reflected in increased peripheral blood values, the ability of these compounds to produce the characteristic polycythemia in rats was studied. This method has previously been used in evaluating the hemopoietic effect of other cobalt compounds (4).

Oral administration of 10 mg./Kg. of elemental cobalt as cobalt polyglucopyranose, as cobalt-niacinamide, or as cobalt chloride produced equivalent increases in all three blood indexes as indicated in Table V. The hemopoietic effect of the two new cobalt compounds is, therefore, equivalent to that of cobalt chloride.

Cobalt disodium ethylenediamine tetra-acetate was administered orally in dosages of 10, 20, and 40 mg./Kg. of elemental cobalt (Table V). The 10 and 20 mg./Kg. dosages did not have any significant effect on the blood indexes. The 40 mg./Kg. dose, however, was equally as effective as cobalt chloride at the 10 mg./Kg. elemental cobalt dose level. This study was continued for five additional months with no further significant increases in the blood indexes.

Both cobalt-niacinamide and cobalt polyglucopyranose were equivalent to cobalt chloride in increasing the blood indexes. Cobalt disodium ethylenediamine tetra-acetate is fully as effective in higher dosages and in view of its lesser toxicity, it has the greatest therapeutic index of any of the cobalt compounds studied.

TABLE IV.—Fe^{59} ERYTHROCYTE INCORPORATION FROM ERYTHROPOIETIN-RICH SERUM PRODUCED BY COBALT ADMINISTRATION

Group	No. Rats	Av. Incorporation, %
Controls	18	8.3
From cobalt chloride treatment	18	19.7
From cobalt polyglucopyranose treatment	13	15.0
From cobalt disodium ethylenediamine tetra acetate treatment	10	17.8

DISCUSSION

Previous studies (3) and data presented in this report show that about 25% of the cobalt present in cobalt chloride is absorbed when given orally to rats at a dosage of 0.25 mg./Kg. elemental cobalt. This dose closely approximates, on a weight basis, the usual human dose. Using labeled (Co^{58}) cobalt polyglucopyranose or labeled (Co^{58}) cobalt disodium ethylenediamine tetra acetate, at the same cobalt dosage level, similar gastrointestinal absorption percentages were evident, as estimated from urinary excretion data.

Chemical tests, *in vitro*, indicate that cobalt polyglucopyranose supplies cobaltous ion in the acid gastric juice but that the complex ion reforms in the alkaline intestinal fluids. The decreased toxicity of this compound argues that absorption is, at least in part, as the complex ion or total molecule rather than as ionic cobalt.

On the other hand, *in vitro* chemical tests indicate that the tightly bound chelate, cobalt disodium ethylenediamine tetra acetate, does not dissociate to liberate free cobalt ions either in acid gastric juice or in alkaline intestinal fluids. It seems probable, therefore, that absorption is that of the undissociated molecule.

TABLE V.—HEMATOLOGIC RESPONSE IN ADULT MALE RATS RECEIVING COBALT COMPOUNDS ORALLY

Compound	Dose mg./Kg. Co	No. Days on Expt.	Erythrocytes million/cu. mm. Initial	Final	Hemoglobin Gm./100 ml. Initial	Final	Hematocrit, % Initial	Final
Cobalt-niacinamide	10	60 (10)[a]	8.7	10.4	14.8	20.6	41.8	54.9
Cobalt chloride	10	60 (9)	8.2	9.9	15.1	21.2	40.4	53.6
Saline		60 (4)	7.1	6.9	13.8	15.7	38.5	41.5
Cobalt PGP[b]	10	55 (8)	7.8	9.9	14.9	18.5	41.2	52.1
Cobalt chloride	10	55 (4)	7.8	10.3	14.7	18.9	41.3	53.3
Saline		44 (4)	8.0	8.2	15.6	15.9	41.6	41.3
Cobalt EDTA[c]	10	55 (8)	7.4	8.3	14.6	17.9	39.9	46.8
Cobalt EDTA	20	55 (6)	7.2	8.8	15.7	19.1	40.8	48.5
Cobalt EDTA	40	55 (6)	7.1	9.1	14.8	19.5	39.9	51.0
Cobalt chloride	10	55 (6)	7.2	9.5	15.2	20.6	40.1	53.4
Saline		55 (5)	7.5	8.3	15.2	17.3	39.9	43.7

[a] Number of animals given in parenthesis. [b] Cobalt polyglucopyranose. [c] Cobalt disodium ethylenediamine tetra acetate.

The similarity of absorption values with free cobalt ion from cobalt chloride, with cobalt complex ion from cobalt polyglucopyranose and with molecular cobalt disodium ethylenediamine tetra-acetate suggests that the absorption mechanism is dependent upon the cobalt atom rather than on the chemical nature of the remainder of the molecule.

It has been reported that ethylenediamine tetra-acetic acid and its alkali salts are poorly absorbed when given orally. Furthermore, the powerful chelating effect of this acidic structure could be expected to bind heavy metal such as cobalt, lead, iron, etc., and carry them unchanged through the gastrointestinal tract. In fact, an excess of this chelating agent appears to act in this fashion. For example, Child (10) reported a decrease in the hemopoietic action of cobalt chloride when administered orally with large dosages of disodium ethylene diamine tetra-acetate in the proportion of 1 to 2 and 1 to 50, and similar findings were obtained by Post (11) after concomitant intraperitoneal and subcutaneous injections of cobalt sulfate and calcium ethylenediamine tetra-acetate, respectively.

On the other hand, it has been shown (12) that the ethylenediamine tetra-acetate chelate compounds of lead, barium, and other similar heavy metals are absorbed from the gastrointestinal tract and our data indicate no inhibition of absorption of the cobalt compound itself.

Variation in the toxicity of cobalt compounds appears to be related, at least in part, to the degree of dissociation of the compound to furnish cobalt ion. Thus, freely ionizing cobalt chloride shows an LD_{50} representing 144 mg./Kg. elemental cobalt; the complex ion from cobalt polyglucopyranose shows a value of more than 328 mg./Kg., the difficultly dissociated cobalt disodium ethylenediamine tetra-acetate gives a value greater than 1,000 mg./Kg. elemental cobalt.

The dissociation of these three compounds can be readily shown to occur in this same order by the addition of ammonium sulfide to their solutions. Precipitation occurs immediately in a solution of the freely ionized cobalt chloride; it is somewhat delayed in solutions of the polyglucopyranose compound and does not occur for twelve hours or longer in solutions of cobalt disodium ethylenediamine tetra-acetate.

As might be expected, the effect of parenteral injection of these compounds appears to follow the same order. Cobalt chloride injections must be carried out very slowly to avoid the systemic "heavy metal effect." Cobalt polyglucopyranose injections may be carried out more rapidly and in greater dosages without toxic effects. Cobalt disodium ethylenediamine tetra-acetate does not produce "heavy metal effects" upon intravenous injection in rabbits even when relatively large dosages are injected.

After absorption, cobalt compounds must either be effective *per se* or must liberate cobalt ion. It has been shown that approximately 15 to 35% of a given dose of cobalt disodium ethylenediamine tetra-acetate appears in the urine as ionic cobalt (8). Since the dissociation constant of this substance shows the cobalt to be very tightly bound, it would be expected that other compounds with greater dissociation constants would be more rapidly and completely broken down. The erythropoietin assay data reported above indicate that significantly greater potency cannot be obtained from a less tightly bound chelate or from a freely ionizing salt since cobalt disodium ethylenediamine tetra-acetate appears to be fully as potent as the less tightly bound compounds. The much lower toxicity of cobalt disodium ethylenediamine tetra-acetate therefore represents a significant improvement in the therapeutic index.

Clinical studies now in progress[1] tend to verify this conclusion and also indicate a marked diminution in gastrointestinal intolerance. The latter might be expected due to the absence of free cobalt ion in the gastric content.

Since cobalt ion is found in the urine after administration of cobalt disodium ethylenediamine tetra-acetate, it is probable that small quantities of ethylenediamine tetra-acetic acid or its disodium salt are formed. Thus, the possibility of toxicity from the latter substances must be considered. Ethylenediamine tetra-acetic acid and its salts do not appear to be very toxic although a few isolated cases of kidney damage have been reported after intravenous injection of large dosages (9). Dosages reported to cause this toxic effect are of the order of 90 mg./Kg. body weight. The administration of cobalt disodium ethylenediamine tetra-acetate in humans would approximate only 6 mg./Kg. a day of the chelate at a dosage of 0.5 Gm. This dosage, even assuming complete dissociation, could provide quantities of the ethylenediamine tetra-acetic acid compound far below the dosage at which toxic symptoms have been reported. In fact, the ethylenediamine tetra-acetic acid salts are generally regarded as relatively nontoxic (13).

[1] For clinical purposes, cobalt polyglucopyranose is identified as Copoietin PGP, cobalt disodium ethylenediamine tetra-acetate is identified as Copoietin A.

Since a portion of the cobalt administered as cobalt disodium ethylenediamine tetra-acetate can be recovered in the urine in ionic form, it was considered desirable to determine whether the free ethylenediamine tetra-acetate formed would chelate with serum calcium and result in calcium deprivation That this dissociated ethylenediamine tetra-acetate in the blood has no significant effect on calcium excretion is evident from the data obtained. The unlikelihood of such an occurrence is evident, of course, when it is noted that ordinary dietary variation could easily cause decrease in calcium intake greater than the theoretical maximum calcium removal, even assuming complete dissociation of the cobalt chelate at the dosage used Our animal studies, especially those on young growing rats, indicate no deleterious effect on either the kidney or other organs In fact, the effect on the kidney appears to be even less significant than that seen following long continued administration of equally high dosages of elemental cobalt administered as cobalt chloride.

The three types of cobalt compounds tested appear to be similar in their ability to enhance the formation of erythropoietin in the rat and this increase in the erythropoietic hormone is reflected in a subsequent increase in peripheral blood values Although larger doses of the chelate were required to produce equivalent hematologic increase, the decreased toxicity of this compound gives a much improved therapeutic index even at the necessary increased dosage level. These facts suggest that temporary high levels of cobalt ion are the cause of cobalt toxicity and that the lower, more prolonged levels given by the slowly dissociating chelate at these large experimental doses, although effective, are much less toxic.

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Dinitrile Formation in the Attempted Acylation of Malonamide and Its Alkyl Derivatives*

By KENNETH J. LISKA and ARVIND P. SHROFF

Attempted acylation of malonamide and mono-alkylmalonamides with acetyl chloride and pyridine indicates that no acylation takes place at room temperature. At elevated temperatures, the predominant reaction is dehydration to the corresponding dinitrile. Only one compound, *n*-butylmalonamide, gave a significant yield of acylated product, identified as N-acetyl-α-cyanocaproamide.

MALONAMIDE and its mono-alkyl derivatives offer two potential sites for the introduction of an acyl group. Treatment of these compounds with the usual reagents might theoretically lead to carbon-acylated products, or nitrogen-acylated products, or both. Liska (1), for example, acylated (2-hydroxy-1-naphthylmethyl)-malonamide with acetyl chloride and pyridine and showed that the product was a diacylated derivative, the second acetyl group having entered at either the methine carbon atom or the amide nitrogen atom.

It appears that no attempts have been made to acylate malonamide or mono-alkylmalonamides with acetyl chloride or other organic acyl halides. Boon and co-workers (2) have acylated C-ethyl-C-phenyl malonamide with acetyl chloride, in a sealed tube, to give an N,N′-diacylated product. Thompson (3) showed that α-chloroacetamide and α,α-dichloroacetamide failed to give any acylated product with different acid chlorides; dehydration was the only observed reaction, indicating that amides which possess electron withdrawing groups on an alpha carbon and which are relatively acidic (compared to acetamide) are less amenable to acylation of any kind and probably more easily dehydrated.

It was the purpose of this investigation to study the behavior of malonamide and certain of its alkyl derivatives when treated with acetyl chloride and pyridine, and to determine, if possible, the preferred site of acylation. Successful completion of this problem might lead to the solution of specific problems involving similar acylations.

EXPERIMENTAL

The following experiment typifies those acylations attempted at a temperature intermediate between room and reflux temperature.

Formation of Ethylmalondinitrile and Ammonium

* Received July 28, 1958, from the School of Pharmacy, Duquesne University, Pittsburgh 19, Pa
Abstracted from a thesis submitted to the Faculty of the Graduate School of Duquesne University by A P Shroff in partial fulfillment of the requirements for the degree of Master of Science

Chloride in the Attempted Acylation of Ethylmalonamide.—Five grams (0.038 mole) of ethylmalonamide, 25 ml. of pyridine, and 2.9 ml. (0.038 mole) of acetyl chloride were heated in a 100-ml. round-bottom flask for one hour at 78-80°. Excess of pyridine was removed and the solid residue was taken up with ethyl alcohol. Filtration followed by recrystallization from 95% acetic acid removed 1.0 Gm. of ammonium chloride. The filtrate, on further distillation, gave ethylmalondinitrile, b. p. 208-209/760 mm. (Lit. 208°/756 mm. (4)). The yield was 2.3 ml. (61%).

Anal.[1]—Calcd. for $C_5H_6N_2$: N, 29.77; Found: N, 29.63.

Acylation of *n*-Butylmalonamide at Reflux Temperature.—Five grams (0.031 mole) of *n*-butylmalonamide contained in a 100-ml. round-bottom flask was dissolved with the aid of heat in 35 ml. of pyridine; 7.2 ml. (0.093 mole) of acetyl chloride was added dropwise to the solution. The solution was heated at reflux temperature for one hour. Precipitation of the acylated derivative was accomplished by concentrating the reaction solution to a volume of 8 ml., chilling, and adding water. Filtration gave a crude product which was recrystallized from water to yield 0.5 Gm. (9%) of white crystals of N-acetyl-α-cyanocaproamide, m. p. 116-117°. The infrared spectrum of the compound in Nujol showed a carbonyl band at 1653 cm.$^{-1}$ and a weak nitrile band at 2203 cm.$^{-1}$. There was no absorption in the ultraviolet.

Anal.[1]—Calcd. for $C_9H_{14}N_2O_2$: C, 59.3, H, 7.7, N, 15.38; Found: C, 59.2, H, 8.7, N, 15.33.

The filtrate on further distillation yielded a yellow liquid, *n*-butylmalondinitrile, b. p. 222-223/760 mm. The yield was 2.6 ml. (62%).

Anal.[2]—Calcd. for $C_7H_{10}N_2$: C, 68.85, H, 8.19, N, 22.95; Found: C, 68.93, H, 8.38, N, 22.85.

Degradation of N-Acetyl-α-cyanocaproamide to Caproamide.—Five hundred milligrams of N-acetyl-α-cyanocaproamide was heated in a 15-ml. round-bottom flask for one hour with 5 ml. of 10% sodium hydroxide solution. The ammonia evolved in the process was detected both by its odor and by litmus paper. Acidification of the solution with hydrochloric acid and chilling gave *n*-butylmalonic acid, m. p. 101-102° (Lit. 101.5-102° (5)). Decarboxylation of the *n*-butylmalonic acid was accomplished by refluxing in 5% hydrochloric acid solution for six hours. A yellowish liquid, presumably caproic acid, was extracted with ether. Four milliliters of thionyl chloride was added to the yellow liquid residue remaining after the removal of the ether and the mixture was refluxed for forty-five minutes. This solution was then added to 40 ml. of aqueous ammonia. Crystals of caproamide separated immediately. Filtration and recrystallization from water yielded pure caproamide, m. p. 101-102°. The mixed m. p. of *n*-butylmalonic acid and caproamide was 99-100°.

[1] Carbon and hydrogen analysis performed by Weiler and Strauss Microanalytical Laboratory, Oxford, England.

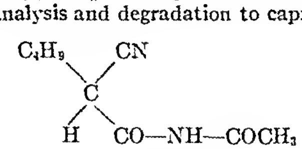
[2] Carbon and hydrogen analysis performed by Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y.

DISCUSSION

The initial acylation reaction for malonamide, ethylmalonamide, propylmalonamide, and butylmalonamide was attempted using acetyl chloride and pyridine at room temperature. Unchanged starting material was recovered in quantitative yield from each reaction. Varying the molar ratio of the amides to acylating agent did not change the results in any case.

At a temperature between room temperature and reflux temperature, each amide mentioned above underwent hydrolysis and dehydration when treated with acetyl chloride and pyridine. The hydrolysis product was ammonium chloride, the yields of which ranged from 19 to 30%; the dehydration product was the corresponding dinitrile ranging in yield from 55 to 67%. Only *n*-propylmalondinitrile was subjected to proof of structure since all of the dinitriles, with the exception of *n*-butylmalondinitrile, are reported in the literature. In each acylation attempted at a temperature between room and reflux temperature, a very small yield of 2,4-dinitrophenylhydrazone derivative was obtained from the crude ammonium chloride. This result was interpreted as an indication of a ketonic compound, and hence of very limited carbon acylation.

At reflux temperatures, malonamide, ethylmalonamide, and propylmalonamide failed to give any acylated product, and since no starting material was recovered, it was presumed that each amide was converted to its corresponding dinitrile. Varying the molar ratio of the amide to the acylating agent did not change the results. However, at reflux temperature, in the presence of acetyl chloride and pyridine, *n*-butylmalonamide underwent N-acylation to give N-acetyl-α-cyanocaproamide (I); the structure of (I) was proved by means of elemental and infrared analysis and degradation to caproamide.



I

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The Synthesis and Pharmacology of Alkyl Esters of N-Substituted Aminoacylaminobenzoic Acids II*

By ELIAS EPSTEIN and DANIEL KAMINSKY

Alkyl esters of N-substituted aminoacetyl-, aminopropionyl-,γ-aminobutyryl-, and δ-aminovalerylaminobenzoic acids were prepared. They, as well as previously described compounds in this series, were tested as salts for anesthetic potency, toxicity, and for irritation on experimental animals. Several of these compounds had a sufficiently high relative anesthetic efficiency (ratio of relative potency to relative toxicity) when compared to procaine hydrochloride to warrant further investigation.

In our previous paper (1) we described several alkyl esters of *o*- and *p*-N-substituted aminoacetyl- and aminopropionylaminobenzoic acids which showed sufficient promise as local anesthetics to warrant clinical trials. Gupta, *et al.* (2), have also prepared several members of this group of compounds. In our continued search for more effective local anesthetics, additional compounds of this type were prepared. The series has been extended to include the *m*-N-substituted aminoacetyl- and the *p*-N-substituted γ-aminobutyryl- and δ-aminovalerylaminobenzoic acid esters. These compounds, as well as those previously described (1), were tested for potency, toxicity, and irritation on laboratory animals.

Table I lists the boiling or melting points and refractive indexes of the nitroesters together with the boiling or melting points, refractive indexes, densities, molecular refractions, and analyses of the corresponding amino esters. Table II lists the chloracylaminobenzoates with their melting points and analyses, while Table III lists the melting points, analyses, and molecular weight determinations of the hydrochlorides of the alkyl esters of the N-substituted aminoacylaminobenzoic acids.

EXPERIMENTAL

The method of preparation consisted of treating a chloroacyl chloride with an ester of aminobenzoic acid and subsequent condensation of the resulting chloranilide with a primary or secondary amine to yield the anesthetic compound, as described in our previous paper (1). The aminobenzoic acid esters not commercially available were prepared by treating the *p*- and *m*-nitrobenzoylchloride with the appropriate alcohol to form the nitroester, which was then reduced catalytically or with iron filings. In the instance of the tertiary amyl ester, the presence of a proton acceptor such as dimethylaniline was necessary to drive the reaction to completion, as suggested by Abramovitch, *et al.* (3), in their preparation of tertiary butyl acetate.

The chloroacetyl, α-chloropropionyl and β-chloropropionyl chlorides were obtained from commercial sources. The γ-chlorobutyryl and δ-chlorovaleryl chlorides were prepared from their respective lactones. Poor yields were obtained by treating these lactones with excess thionyl chloride at elevated temperatures. Considerably better results were obtained using a zinc chloride catalyst, as suggested by a German patent (4).

Low yields were obtained for the chlorobutyryl and chlorovaleryl anilines by the method used and previously described (1) for the preparation of the chloroacetyl- and chloropropionylanilines. The use of a proton acceptor, such as dimethylaniline with a modified procedure, gave excellent yields.

t-Amyl *p*-Nitrobenzoate.—A solution of 205 Gm. (1.1 mole) of *p*-nitrobenzoyl chloride in 700 ml. of dry benzene was added, dropwise, over a period of one hour, to a stirred solution of 88 Gm. (1.0 mole) of *t*-amyl alcohol in 121 Gm. (1.0 mole) of freshly distilled dimethylaniline. The mixture was stirred for an additional hour and then refluxed for two hours. After cooling, the mixture was successively washed with three 100-ml. portions of 5% hydrochloric acid, two 100-ml. portions of 10% sodium hydroxide, and two 100-ml. portions of water. The benzene was removed by distillation and the residue distilled; b. p. 90–92° (25μ) yield 187 Gm. (79%). Recrystallization from isopropyl alcohol yielded light yellow crystals, m. p. 54–56°.

γ-Chlorobutyryl Chloride.—A solution of 129 Gm. (1.5 mole) γ-butyrolactone and 5 Gm. anhydrous zinc chloride in 197 Gm. (1.65 mole) thionyl chloride was maintained at 50–60° for two hours. After twelve hours at room temperature the solution was distilled; b. p. 73–74° (15 mm.), lit. (4) 76–77° (18 mm.); yield 117 Gm. (55%) as a colorless oil.

Butyl Ester of *p*-(γ-Chlorobutyrylamino)-benzoic Acid.—To a stirred solution of 55 Gm. (0.3 mole) of butyl *p*-aminobenzoate in 500 ml. ether was added 50 Gm. (0.35 mole) of γ-chlorobutyryl chloride. After stirring the resulting thick slurry for one hour, 48 Gm. (0.4 mole) of N,N-dimethylaniline in 200 ml. ether was added, dropwise, keeping the temperature below 38° with continued stirring over a period of one-half hour. The mixture was stirred for an additional hour and then extracted with four 250-ml. portions of 10% hydrochloric acid and once with water. Evaporation of the ether layer yielded 79 Gm. of a light yellow product. Recrystallization from benzene yielded 70 Gm. (79%), m. p. 91–92°, as white crystals.

* Received July 19, 1958, from the Research Laboratories of Novocol Chemical Mfg. Co., Inc., Brooklyn, N. Y.
The authors are indebted to Richard Sriubas for assistance in the analyses and to Ann Hartman for assistance in the pharmacological testing of these compounds.

Table I.—Alkyl Nitro- and Aminobenzoates[a]

Alkyl	Position	Nitro Esters b. p., ° C., (m. p.)	Nitro Esters n_D^{20}	Amino Esters b. p., ° C., (m. p.)	Amino Esters n_D^{20}	Amino Esters d_{20}^{20}	MR, Theory	MR, Found	Formula	Nitrogen, % Theory	Nitrogen, % Found
C_2H_5	*m*	(43–44)[b]	...	84–85/20µ[c]	1.5499	1.173	46.12	45.33	$C_9H_{11}O_2N$	8.48	8.31
i-C_3H_7	*m*	77–78/20µ	1.5210	97–99/20µ	1.5436	1.090	50.74	51.95	$C_{10}H_{13}O_2N$	7.83	7.78
C_4H_9	*m*	90–92/25µ	1.5215	95–96/20µ[d]	1.5424	1.074	55.36	56.80	$C_{11}H_{15}O_2N$	7.25	7.09
i-C_4H_9	*m*	86–87/30µ	1.5179	93–94/25µ	1.5399	1.065	55.36	56.68	$C_{11}H_{15}O_2N$	7.25	7.15
C_5H_{11}	*m*	101–102/30µ	1.5171	106–107/25µ	1.5362	1.054	59.98	61.21	$C_{12}H_{17}O_2N$	6.76	6.86
s-C_5H_{11}	*m*	93–95/20µ	1.5162	97–98/30µ	1.5322	1.042	59.98	61.65	$C_{12}O_{17}O_2N$	6.76	6.63
t-C_5H_{11}	*p*	90–92/25µ (54–56)	...	(89–91)	...	...	...	...	$C_{12}H_{17}O_2N$	6.76	6.60
C_6H_{13}	*p*	108–110/20µ[e]	1.5156	(62–64)	...	...	...	...	$C_{13}H_{19}O_2N$	6.33	6.18

[a] Amino esters used as intermediates and not recorded here are commercially available or have been previously prepared by us (1).
[b] Reported (5) 43°.
[c] Reported (6) b. p. 291°.
[d] Reported (7) b. p. 215°.
[e] Reported (8) b. p. 175–177° at 8 mm n_D^{20} 1.5153.

PHARMACOLOGY

A pharmacological screening of these compounds for use as local anesthetics was conducted and the results of the more promising compounds noted in Table IV. The hydrochloride salt of about one-third of these compounds was too insoluble and the gluconate salt was substituted. Since the anesthetic potency and toxicity are primarily functions of the concentration of the anesthetic base, the gluconate salt was evaluated on the basis of the equivalent amount of the hydrochloride.

Evidences of irritation were checked after topical application to the eye and on intradermal injection of the rabbit. The toxicity was determined on white mice, using both the intraperitoneal and subcutaneous mode of injection. Potency was determined by: blocking the sciatic nerve of the intact guinea pig for conduction anesthesia, application to the rabbit eye for topical anesthesia, and with the wheal test on the guinea pig for infiltration anesthesia. A detailed description of these tests has been previously published (9).

To compare the value of these compounds as local anesthetics with each other and with procaine hydrochloride, we used the quotient of the relative potency as determined by the guinea pig wheal test to the relative toxicity as determined by the intraperitoneal injection on white mice. The guinea pig wheal test for anesthetic potency gives a close correlation to clinical findings with commercially used anesthetics (10). The intraperitoneal toxicity on white mice approximates a mean value between the intravenous toxicity, where absorption of the drug is rapid, and the subcutaneous toxicity, where absorption is delayed.

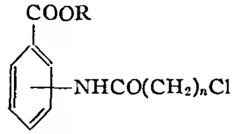
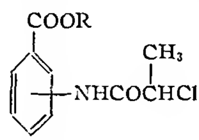
Since we were screening a rather large number of compounds for their potential value as local anesthetics, a minimum number of animals was used, and the values listed in Table IV should be considered as only semiquantitative. For the determination of potency in the rabbit eye, an average of four eyes was used for each compound. An average of eight wheals and six legs was used for the wheal and sciatic nerve tests, respectively, on the guinea pig. The subcutaneous and intraperitoneal toxicities were conducted at three critical dose levels, using three animals at each level. The critical dose is one near or at the LD_{50}. In describing irritation, 0 stands for little or no irritation, + for moderate irritation, ++ severe irritation. The detailed description of these levels of irritation is noted in the literature (9, 11).

DISCUSSION AND SUMMARY

Sixty alkyl esters of N-substituted aminoacylaminobenzoic acids were prepared. They, together with sixty-nine similar compounds previously reported, were tested pharmacologically for potential value as local anesthetics.

Although a few general rules have been postulated correlating physiological activity of benzoic acid esters with their molecular structure (12), little light has been shed on this relationship with anilides. In either case, it has

TABLE II —CHLOROACYLAMINOBENZOATES[a]

R	Position	n	M p , ° C	Formula	Chlorine, % Calcd	Chlorine, % Found
						
C_2H_5	*m*	1	81–82	$C_{11}H_{12}O_3NCl$	14 67	14 49
C_2H_5	*p*	3	98–99	$C_{13}H_{16}O_3NCl$	13 15	13.05
i-C_3H_7	*m*	1	98–100	$C_{12}H_{14}O_3NCl$	13 87	13 77
C_4H_9	*m*	1	68–70	$C_{13}H_{16}O_3NCl$	13 15	12 99
C_4H_9	*p*	3	91–92	$C_{15}H_{20}O_3NCl$	11 91	11.78
C_4H_9	*p*	4	63–64	$C_{16}H_{22}O_3NCl$	11 37	11 38
i-C_4H_9	*m*	1	96–97	$C_{13}H_{16}O_3NCl$	13 15	13 18
i-C_4H_9	*p*	3	135–136	$C_{15}H_{20}O_3NCl$	11 91	11 79
C_5H_{11}	*m*	1	65–67[b]	$C_{14}H_{18}O_3NCl$	12 50	12 41
C_5H_{11}	*p*	2	87–88	$C_{15}H_{20}O_3NCl$	11 91	11 96
s-C_5H_{11}	*m*	1	[c]	$C_{14}H_{18}O_3NCl$	12 50	12 57
t-C_5H_{11}	*p*	1	114–116	$C_{14}H_{18}O_3NCl$	12 50	12 38
C_6H_{13}	*p*	1	81–83	$C_{15}H_{20}O_3NCl$	11 91	11 79
						
C_2H_5	*o*		44–45[d]	$C_{12}H_{14}O_3NCl$	13.87	13 89
C_4H_9	*p*		75–77	$C_{14}H_{18}O_3NCl$	12 50	12 38

[a] The chloroacylaminobenzoates used as intermediates and not recorded here were previously prepared by us (1) [b] B. p 166–168°/60μ [c] B p 165–167°/25μ, n_D^{20} 1 5129 [d] B p 115–116°/60μ.

been difficult to predict the usefulness of a compound as a local anesthetic from its structure. On screening these alkyl aminoacylaminobenzoates, we found that this uncertainty persisted.

The relative local anesthetic efficiency, the quotient of the relative potency to the relative toxicity, can be affected by many unrelated phenomena. Rates of absorption and detoxification, solubility at the pH of the body, vasomotory effect on the capillaries, and other obscure effects play an important role. We noted that some of our compounds precipitated at body pH and in many cases this appeared to be a direct cause of irritation, lower toxicity, and longer duration of the anesthetic effect. Since precipitation occurred more readily at higher concentrations, the guinea pig wheal test had an advantage over the sciatic nerve test, as the concentrations used were only one-fifth as great.

For the purpose of correlating molecular structure to physiological activity, we used the relative intraperitoneal toxicity in white mice, the relative potency as determined by the guinea pig wheal test, and the irritation as observed on both the rabbit eye and skin. The following generalizations can be made:

1. Effect of varying the ester group (R). (*a*) Potency and irritation increased with increasing molecular weight, with the straight chains being more potent and irritating than the branched chains. The methyl ester compounds were exceptions, being more potent than the ethyl esters. (*b*) Molecular size or branching of the ester group had little effect on toxicity. (*c*) Efficiency increased with increasing molecular weight and reached a maximum at five carbon atoms.

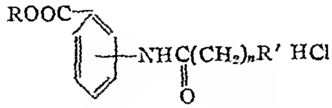
2. Effect of varying the relative position of the ester with the anilide group (*ortho*, *meta*, *para*). Although the *meta* derivatives of the aminobenzoic acid esters have been reported (13) to be much less toxic and more efficient than the corresponding *para* derivatives, this group of compounds had a reverse relationship. (*a*) With respect to potency and irritation, the *meta*>*ortho*>*para*. (*b*) With respect to toxicity and anesthetic efficiency, the *ortho*>*meta*>*para*.

3. Effect of varying the acyl chain. (*a*) Branched chain acyl compounds were slightly less toxic and irritating, equal in potency and greater in efficiency than the straight chain acyl compounds of the same number of carbon atoms in the chain. (*b*) Increasing the length of the acyl chain (*n*) increased the irritation and decreased the anesthetic efficiency.

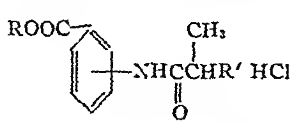
We were not able to obtain a clearcut relationship of *n* with respect to potency and toxicity.

4. Effect of varying the terminal alkylamino

TABLE III.—ALKYL ESTERS OF N-SUBSTITUTED AMINOACYLAMINOBENZOIC ACID HYDROCHLORIDES

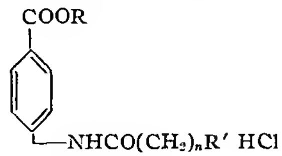


R	R′	n	Position	M. p., °C	Formula	Chlorine, % Calcd	Chlorine, % Found	Mol. wt. Calcd	Mol. wt. Found
C_2H_5	NHC_2H_5	1	*o*	205–207	$C_{13}H_{19}O_3N_2Cl$	12.36	12.22	287	281
C_2H_5	NHC_2H_5	2	*o*	167–168	$C_{14}H_{21}O_3N_2Cl$	11.79	11.70	301	297
C_2H_5	NHC_3H_7	1	*o*	174–176	$C_{14}H_{21}O_3N_2Cl$	11.79	11.82	301	301
C_2H_5	NHC_3H_7	2	*o*	169–171	$C_{15}H_{23}O_3N_2Cl$	11.26	11.15	315	321
C_2H_5	NHC_3H_7(iso)	1	*o*	172–175	$C_{14}H_{21}O_3N_2Cl$	11.79	11.75	301	299
C_2H_5	NHC_3H_7(iso)	2	*o*	185–186	$C_{15}H_{23}O_3N_2Cl$	11.26	11.31	315	322
C_2H_5	NHC_4H_9(iso)	1	*m*	211–213	$C_{15}H_{23}O_3N_2Cl$	11.26	11.20	315	314
C_2H_5	NHC_4H_9(iso)	3	*p*	174–176	$C_{17}H_{27}O_3N_2Cl$	10.34	10.48	343	
C_2H_5	$N(C_2H_5)_2$	1	*m*	122–124	$C_{15}H_{23}O_3N_2Cl$	11.26	11.22	315	313
C_2H_5	$N(C_2H_5)_2$	3	*p*	152–154	$C_{17}H_{27}O_3N_2Cl$	10.34	10.16	343	
C_2H_5	Morpholino	1	*m*	199–201	$C_{15}H_{21}O_4N_2Cl$	10.75	10.89	329	327
i-C_3H_7	NHC_2H_5	1	*m*	208–209	$C_{14}H_{21}O_3N_2Cl$	11.79	11.75	301	309
i-C_3H_7	NHC_4H_9	1	*m*	198–200	$C_{16}H_{25}O_3N_2Cl$	10.78	10.74	329	329
i-C_3H_7	NHC_4H_9(iso)	1	*m*	218–220	$C_{16}H_{25}O_3N_2Cl$	10.78	11.02	329	331
i-C_3H_7	NHC_4H_9(tert)	1	*m*	250–252	$C_{16}H_{25}O_3N_2Cl$	10.78	10.72	329	325
i-C_3H_7	$N(C_2H_5)_2$	1	*m*	147–149	$C_{16}H_{25}O_3N_2Cl$	10.78	10.91	329	321
i-C_3H_7	Morpholino	1	*m*	200–201	$C_{16}H_{23}O_4N_2Cl$	10.34	10.40	343	344
C_4H_9	NHC_4H_9(iso)	1	*m*	168–169	$C_{17}H_{27}O_3N_2Cl$	10.34	10.47	343	337
C_4H_9	NHC_4H_9(iso)	3	*p*	184–185	$C_{19}H_{31}O_3N_2Cl$	9.56	9.48	371	379
C_4H_9	NHC_4H_9(iso)	4	*p*	198–199	$C_{20}H_{33}O_3N_2Cl$	9.21	9.15	385	394
C_4H_9	$N(C_2H_5)_2$	1	*m*	99–102	$C_{17}H_{27}O_3N_2Cl$	10.34	10.18	343	334
C_4H_9	$N(C_2H_5)_2$	3	*p*	155–156	$C_{19}H_{31}O_3N_2Cl$	9.56	9.48	371	
C_4H_9	$N(C_2H_5)_2$	4	*p*	122–126	$C_{20}H_{33}O_3N_2Cl$	9.21	9.34	385	
C_4H_9	Morpholino	1	*m*	191–194	$C_{17}H_{25}O_4N_2Cl$	9.93	9.89	357	354
i-C_4H_9	NHC_2H_5	1	*m*	129–134	$C_{15}H_{23}O_3N_2Cl$	11.26	11.45	315	308
i-C_4H_9	NHC_2H_5	2	*p*	175–176	$C_{16}H_{25}O_3N_2Cl$	10.78	10.70	329	330
i-C_4H_9	NHC_3H_7	1	*m*	196–198	$C_{16}H_{25}O_3N_2Cl$	10.78	10.63	329	324
i-C_4H_9	NHC_4H_9(iso)	1	*m*	201–203	$C_{17}H_{27}O_3N_2Cl$	10.34	10.27	343	338
i-C_4H_9	NHC_4H_9(iso)	3	*p*	187–188	$C_{19}H_{31}O_3N_2Cl$	9.56	9.42	371	382
i-C_4H_9	$N(C_2H_5)_2$	1	*m*	134–136	$C_{17}H_{27}O_3N_2Cl$	10.34	10.31	343	339
i-C_4H_9	Piperidino	1	*m*	152–154	$C_{18}H_{27}O_3N_2Cl$	10.00	9.89	355	357
C_5H_{11}	$NHCH_3$	1	*p*	171–173	$C_{16}H_{23}O_3N_2Cl$	11.26	11.12	315	309
C_5H_{11}	NHC_2H_5	1	*m*	166–168	$C_{16}H_{25}O_3N_2Cl$	10.78	10.99	329	322
C_5H_{11}	NHC_2H_5	2	*p*	194–196	$C_{17}H_{27}O_3N_2Cl$	10.34	10.18	343	343
C_5H_{11}	NHC_3H_7	1	*m*	169–170	$C_{17}H_{27}O_3N_2Cl$	10.34	10.23	343	339
C_5H_{11}	NHC_3H_7	1	*p*	212–214	$C_{17}H_{27}O_3N_2Cl$	10.34	10.30	343	343
C_5H_{11}	NHC_4H_9	1	*m*	169–172	$C_{18}H_{29}O_3N_2Cl$	9.93	9.84	357	355
C_5H_{11}	NHC_4H_9(iso)	1	*m*	162–164	$C_{18}H_{29}O_3N_2Cl$	9.93	10.02	357	352
C_5H_{11}	NHC_4H_9(iso)	2	*p*	181–183	$C_{19}H_{31}O_3N_2Cl$	9.56	9.62	371	369
C_5H_{11}	NHC_4H_9(tert)	1	*p*	245–247	$C_{18}H_{29}O_3N_2Cl$	9.93	9.96	357	357
C_5H_{11}	NHC_5H_{11}	1	*p*	260–263	$C_{19}H_{31}O_3N_2Cl$	9.56	9.56	371	363
C_5H_{11}	$N(C_2H_5)_2$	1	*m*	107–109	$C_{18}H_{29}O_3N_2Cl$	9.93	9.88	357	355
C_5H_{11}	$N(C_2H_5)_2$	2	*p*	124–127	$C_{19}H_{31}O_3N_2Cl$	9.56	9.63	371	368
C_5H_{11}	Morpholino	1	*m*	186–189	$C_{18}H_{25}O_4N_2Cl$	9.56	9.66	371	375
s-C_5H_{11}	NHC_3H_7	1	*m*	172–174	$C_{17}H_{27}O_3N_2Cl$	10.34	10.42	343	342
s-C_5H_{11}	NHC_4H_9(iso)	1	*m*	170–172	$C_{18}H_{29}O_3N_2Cl$	9.93	9.87	357	352
s-C_5H_{11}	Morpholino	1	*m*	171–174	$C_{18}H_{27}O_4N_2Cl$	9.56	9.42	371	369
t-C_5H_{11}	NHC_3H_7	1	*p*	225–228	$C_{17}H_{2}\ O_3N_2Cl$	10.34	10.26	343	337
t-C_5H_{11}	$N(C_2H_5)_2$	1	*p*	227–229	$C_{18}H_{29}O_3N_2Cl$	9.93	9.90	357	356
t-C_5H_{11}	Morpholino	1	*p*	182–184	$C_{18}H_{27}O_4N_2Cl$	9.56	9.43	371	362
C_6H_{13}	NHC_3H_7	1	*p*	284–286	$C_{18}H_{29}O_3N_2Cl$	9.93	9.85	357	353
C_6H_{13}	NHC_3H_7(iso)	1	*p*	242–244	$C_{18}H_{29}O_3N_2Cl$	9.93	9.90	357	351
C_6H_{13}	NHC_4H_9(iso)	1	*p*	213–215	$C_{19}H_{31}O_3N_2Cl$	9.56	9.73	371	374
C_6H_{13}	$N(C_2H_5)_2$	1	*p*	178–182	$C_{19}H_{31}O_3N_2Cl$	9.56	9.41	371	364

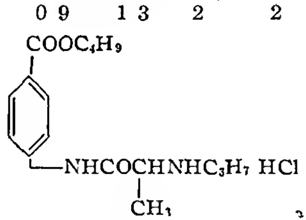


R	R′	n	Position	M. p., °C	Formula	Chlorine, % Calcd	Chlorine, % Found	Mol. wt. Calcd	Mol. wt. Found
C_2H_5	NHC_4H_9(iso)		*o*	114–117	$C_{16}H_{25}O_3N_2Cl$	10.78	10.69	329	323
C_2H_5	Morpholino		*o*	190–193	$C_{16}H_{23}O_4N_2Cl$	10.34	10.24	343	338
C_4H_9	NHC_3H_7		*p*	175–177	$C_{17}H_{27}O_3N_2Cl$	10.34	10.31	343	346
C_4H_9	NHC_4H_9(iso)		*p*	160–162	$C_{18}H_{29}O_3N_2Cl$	9.93	9.99	357	359
C_4H_9	$N(C_2H_5)_2$		*p*	147–149	$C_{18}H_{29}O_3N_2Cl$	9.93	10.10	357	360
C_4H_9	Morpholino		*p*	172–174	$C_{18}H_{27}O_4N_2Cl$	9.56	9.47	371	370

TABLE IV.—PHARMACOLOGY OF ALKYL ESTERS OF N-SUBSTITUTED AMINOACYLAMINOBENZOIC ACID HYDROCHLORIDES



R	R′	n	Position	Relative Potency: Rabbit eye[a]	Relative Potency: Guinea pig[b] Wheal	Guinea pig[b] Sciatic Nerve: No Ep	Sciatic Nerve: W Ep	Relative Toxicity, i.p.	Relative Toxicity, s.c.	Irritation: Eye	Irritation: Skin	Relative Efficiency
C_2H_5	NHC_3H_7(iso)	1	*o*	1.1	2.4	1	0.1	0.4	0.5	+	+	6.0
C_2H_5	NHC_4H_9	2	*o*	2.4	10	3	4	0.8	0.9	+	0	13
C_2H_5	NHC_4H_9(iso)	1	*o*	0.1	1.3	6	2	0.2	0.2	0	++	6.5
C_2H_5	$N(C_2H_5)_2$	1	*o*	0.2	1.9	3	4	0.4	0.5	+	+	4.8
C_2H_5	Cyclohexylamino	1	*p*	0.4	1.5	1	3	0.1	<0.3[c]	0	+	15
i-C_3H_7	NHC_3H_7	2	*p*	3.8	3.0	4	1	0.8	0.6	++	0	3.8
i-C_3H_7	NHC_4H_9(iso)	1	*m*	4.0	6.4	2	1	0.9	0.8	+	0	7.1
C_4H_9	NHC_4H_9	2	*p*	0.9	1.1	3	1	0.3	0.2	+	0	3.7
C_4H_9	Morpholino	2	*p*	0.9	1.3	2	1	0.2	0.3	+	0	6.5
i-C_4H_9	NHC_4H_9(iso)	2	*p*	1.7	1.9	0.9	0.8	0.4	0.3	+	0	4.8
i-C_4H_9	$N(C_2H_5)_2$	1	*m*	1.0	2.5	1	0.4	0.1	<0.2	0	++	25
i-C_4H_9	$N(C_2H_5)_2$	2	*p*	0.5	2.5	3	2	0.1	<0.2	0	0	25
i-C_4H_9	Morpholino	1	*p*	0.8	1.1	2	0.5	0.1	0.1	+	0	11
i-C_4H_9	Morpholino	2	*p*	1.0	1.0	3	2	0.1	<0.2	+	0	10
i-C_4H_9	Cyclohexyl-methylamino	1	*p*	1.6	1.9	5	1	0.3	0.3	0	+	6.3
i-C_4H_9	Benzylamino	1	*p*	0.3	1.9	4	1	0.1	0.2	0	+	19
C_5H_{11}	NHC_2H_5	1	*m*	1.2	7.6	2	0.5	1.5	1.9	+	0	5.1
C_5H_{11}	NHC_2H_5	1	*p*	1.2	5.1	2	4	0.4	<0.3	+	0	13
C_5H_{11}	NHC_3H_7	1	*p*	1.8	1.5	2	1	0.3	0.3	+	+	5.0
C_5H_{11}	NHC_4H_9(iso)	1	*m*	1.0	4.8	1	1	0.6	0.3	+	+	8.0
C_5H_{11}	NHC_4H_9(tert)	1	*p*	2.5	1.0	0.9	0.7	0.2	0.2	+	0	5.0
C_5H_{11}	$N(C_2H_5)_2$	1	*p*	0.9	1.3	2	2	0.1	0.2	+	+	13



				1.0	1.5	1	1	0.4	0.5	0	+	3.8

[a] Cocaine = 1 [b] Procaine = 1 [c] Values of < no used when solubility was too low to obtain the final value.

group (R′). There was little apparent correlation between the structure of this group and physiological activity other than the tertiary alkylamino groups being less toxic than the corresponding secondary group with the same number of carbon atoms.

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Kinetics of Formation of Anhydrovitamin A From Vitamin A Alcohol and Its Acetate*

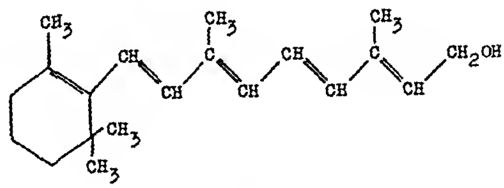
By TAKERU HIGUCHI and JEROME A. REINSTEIN†

Of the several pathways by which vitamin A and its derivatives can degrade, the present study is concerned with those leading to the formation of anhydrovitamin A. Results indicate (*a*) anhydro formation does not occur readily with vitamin A alcohol in the absence of a strong catalyst such as hydrogen chloride, (*b*) the reaction proceeds at a significant rate for solutions of the acetate in both alcoholic and hydroalcoholic systems, (*c*) the conversion of the acetate to the anhydro form is much more rapid in the presence of water than in its absence, and (*d*) formation from the acetate does not occur in ether or hydrocarbon solvent in the absence of catalytic agents. Catalytic behavior of hydrogen chloride, perchloric acid, and acetic acid were also studied. The results on experiments made with pyridine and sodium hydroxide as possible inhibitors are also presented.

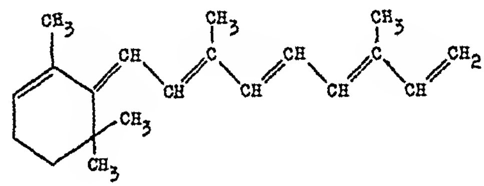
Although from a pharmaceutical and economic standpoint reactions responsible for loss in activity of vitamin A and its derivatives are of considerable importance, relatively little basic information is available concerning the mechanism of breakdown of this vitamin under practical conditions. The facts that we do have, however, point to several broad general class reactions which may be responsible for the observed instability of the polyenoid vitamin. These are (*a*) free radical mediated oxidation and polymerization reactions, (*b*) photochemical reactions, (*c*) isomerization, (*d*) addition reactions involving the vitamin's double bonds and protonated solvents, and (*e*) elimination reactions resulting in the formation of anhydrovitamin A. The present communication is concerned with the results of an investigation into some aspects of the fifth route of breakdown.

Since vitamin A is oftentimes formulated in vehicles containing alcoholic solvents, this study has been directed largely toward evaluation of several factors which may govern the relative rate of the elimination reaction in various hydroxylic and nonhydroxylic solvents. Some earlier work on the hydrogen chloride catalyzed reaction has been repeated and expanded.

The tendency of vitamin A and its derivatives to form anhydrovitamin A (Fig. 1) can be readily followed by ultraviolet spectrophotometry. The increase in length of the conjugated chain results in the appearance of a series of absorption peaks in the near ultraviolet in a region partly transparent for the parent compound. Fortunately



All-trans Vitamin A_1



Anhydrovitamin A

Figure 1.

the absorption spectrum of pure anhydrovitamin A has already been determined by Shantz and colleagues (1). Thus by following the increase in the absorbance of the degrading system at or near 392 mμ, the long wavelength absorption peak for the anhydro form, the kinetics of the reaction can be ascertained easily. In the present studies the long wavelength peak appeared at 386.5 mμ (Fig. 2). Apparently the displacement of the peak was caused by the absorbance of the other products present. The peak has previously been found to appear at 388 mμ (2) and 389 mμ (3).

PAST WORK

Anhydrovitamin A was first reported as a product of the action of anhydrous hydrogen chloride on vitamin A in ethanol by Edisbury, *et al* (4). They noted that this treatment resulted in the appearance of narrow bands in the ultraviolet absorption spectrum. For many years the nature of the transformation was misinterpreted and the compound was known as "cyclized vitamin A" (5). In 1943 Shantz, Cawley,

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From a thesis submitted by Jerome A. Reinstein to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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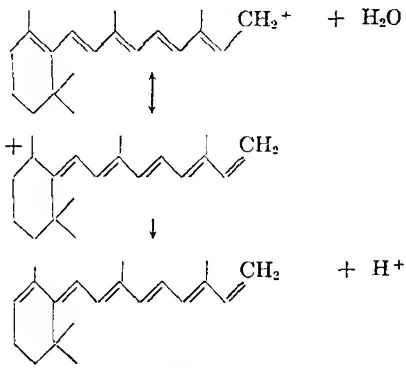
and Embree (1), and Meunier, Dulou, and Vinet (6) presented evidence that the substance was formed not by cyclization but by loss of a molecule of water from vitamin A alcohol. The compound was renamed anhydrovitamin A. It had 0.4% of the biological activity of vitamin A.

Meunier, *et al.* (6), suggested a mechanism for proton catalyzed formation of anhydrovitamin A which seems quite plausible:

Vit. A alcohol + $H^+ \rightarrow$



Anhydrovitamin A

A compound which appeared to be an addition product of vitamin A and hydrogen chloride was obtained by Guerillot-Vinet, Meunier, Jouanneteau, and Gourevitch (7). They confirmed the observation of Shantz and colleagues (1) that prolonged action of hydrogen chloride transformed anhydrovitamin A into "isoanhydrovitamin A" and found that the action of hydrogen chloride did not stop there but caused new spectral changes in which the three bands of "isoanhydrovitamin A" were replaced by a single band at 325 mμ nearly superimposable on that of vitamin A itself. The compound, which they called "substance X," was biologically inactive.

In 1954 Oroshnik (8) proposed that "isoanhydrovitamin A" was in fact not an isomer of anhydrovitamin A, but an ethoxy addition product formed by solvation of the terminal vinyl group of anhydrovitamin A by ethanol.

The conversion of vitamin A to anhydrovitamin A has been studied as a means of determining the vitamin in natural products (9). Robeson and Baxter (10) in the course of their study of the properties of neovitamin A, compared the rates of dehydration of vitamin A and its neo isomer and found that the neo form dehydrated more slowly.

OBSERVATIONS

The elimination reaction responsible for formation of anhydrovitamin A appears to be rather sensitive to experimental conditions. The free vitamin alcohol, for example, has been shown in a previous study (11) to be highly resistant toward this reaction. Despite the considerably greater susceptibility of the alcohol toward oxidative and other free radical mediated losses, it exhibits such great resistance toward dehydration in the absence of catalysts that the reaction could only be studied conveniently in their presence.

In the present report results are given in the first part on the essentially uncatalyzed elimination reaction as occurring in several solvent systems. Since only the ester undergoes this reaction readily, the investigation was confined to this form. In the second part, the elimination behavior of both the ester and the alcohol in the presence of catalytic agents and additives is discussed.

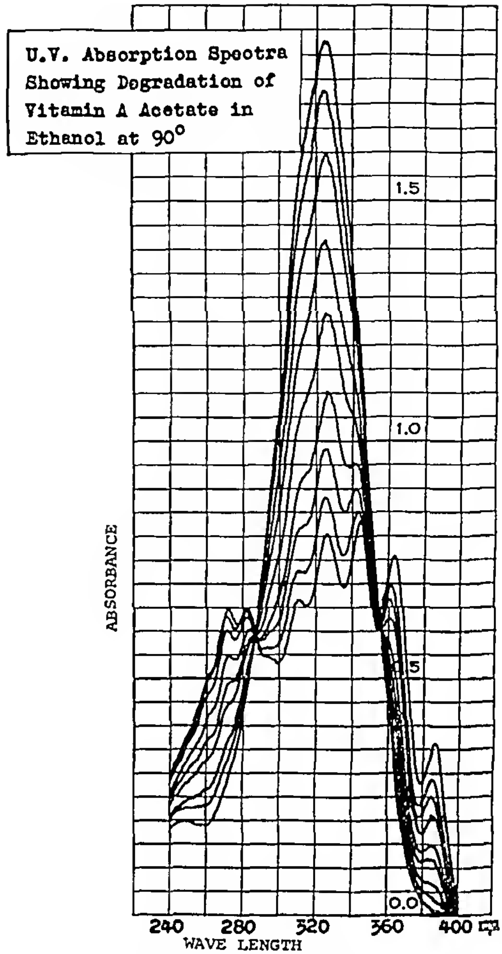


Fig. 2.—Vitamin A peak at 326 mμ decreases with time. Anhydrovitamin A peaks at 350, 367, and 387 mμ increase with time. Vitamin A epoxide peaks between 270 and 285 mμ increase with time.

Anhydrovitamin A Formation From Vitamin A Acetate in Several Solvents

Order of the Reaction with Respect to Substrate.—Though any simple mechanism leading to the formation of anhydrovitamin A by an elimination reaction would yield a first order dependency on the concentration of the initial compound, results such as shown in Fig. 3 indicated that the overall process is quite complex and may not necessarily show a first order behavior. In this plot the percentage of anhydro compound formed with respect to the initial amount of vitamin A acetate is shown as a function of time in a hydroalcoholic medium at 25°. If simple first order kinetics were being obeyed, the curve would follow the usual mathematical form

$$\text{per cent conversion} = 100(1 - e^{-kt}).$$

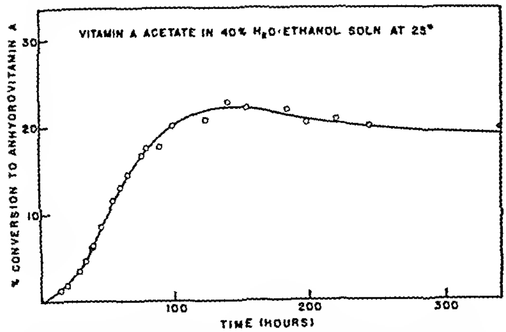


Fig. 3.—Conversion of vitamin A acetate (60 mg. per liter) in 40% ethanol: water to anhydrovitamin A at 25°.

The second derivative would always be negative. This obviously is not the case.

The exact rate dependency on the substrate concentration can be determined more directly. A series of runs were made on the acetate at varying initial concentrations as shown in Fig. 4. The initial rates of formation of the anhydro species, as calculated from the curves, were plotted against the initial concentrations of acetate. The resulting plot is shown in Fig. 5. The straight line passing through the origin indicates that the reaction is first order with respect to the substrate as expected. The particular runs were made in 40% water in ethanol, but similar results were also found in pure ethanol as shown in Fig. 6.

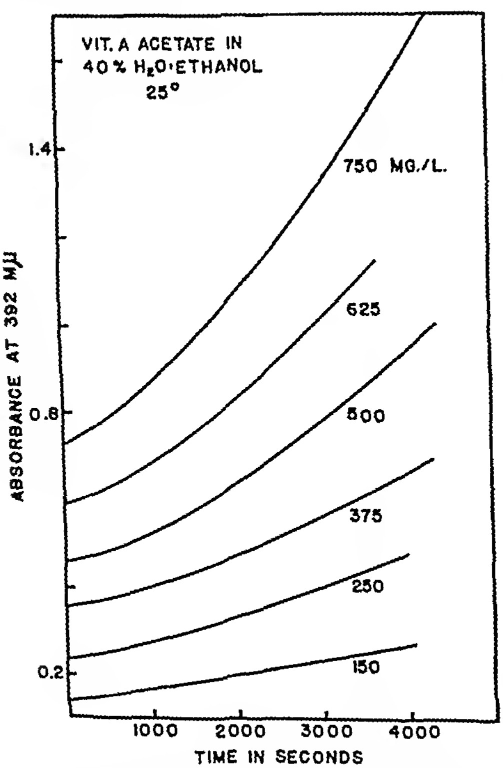


Fig. 4.—Formation of anhydrovitamin A from varying concentrations of vitamin A acetate in 40% water: ethanol at 25°. Rate of formation of peak at 392 mμ.

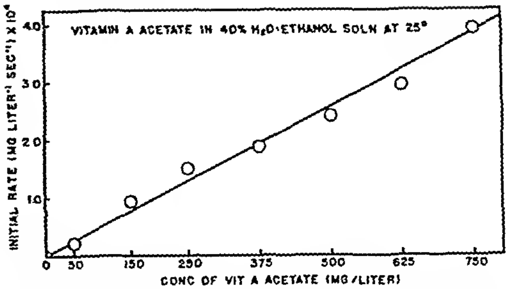


Fig. 5.—Dependence of initial rate of formation of anhydrovitamin A on concentration of vitamin A acetate in 40% water: ethanol at 25°.

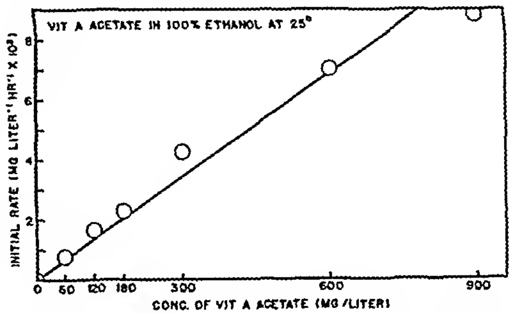


Fig. 6.—Dependence of initial rate of formation of anhydrovitamin A on concentration of vitamin A acetate in pure ethanol at 25°.

Dependence of the Rate of Formation of Anhydrovitamin A on the Solvent Composition.—The rate of formation of the anhydro form from the ester appears to be greatly dependent on the nature of the solvent used in carrying out the reaction. In the absence of a hydroxylic solvent the rate is reduced to negligible proportions. Studies in heptane and isopropyl ether made in connection with this investigation indicated no trace of the reaction, all breakdown apparently occurring through oxidation or related routes.

That anhydrovitamin A could be obtained from vitamin A ester in ethanol was noted previously by Gray and Cawley (11) in 1942. Based on their observations Shantz and colleagues (1) found that when "vitamin A ester concentrate is refluxed. . .with. . .a lower alkyl alcohol . . . the vitamin A ester breaks down into anhydrovitamin A, free fatty acid, and other products." At 25° in 100% ethanol the present study showed that the rate of conversion of vitamin A acetate to the anhydro form was approximately 1.6×10^{-3}% per hour (Fig. 7).

For reactions run in hydroalcoholic solutions, the rate of formation of anhydrovitamin A was found to increase very rapidly as the per cent water in ethanol increased. Data for the variation in initial rate of anhydro A formation with concentration of water in ethanol are show- -log plot in Fig. 8. The rate in ubtracted from the total rate to give the net rate due to water. The slope of the resulting curve at any water concentration, it is evident, is equal to the order of the reaction with respect to water at that concentration.

It can be seen that the effect of water is rather complex since the order is constantly changing. From much less than one at 2% water, the order increases to one at 5%, and then to two at 20%. At 30% the slope appears to incline sharply, and the or-

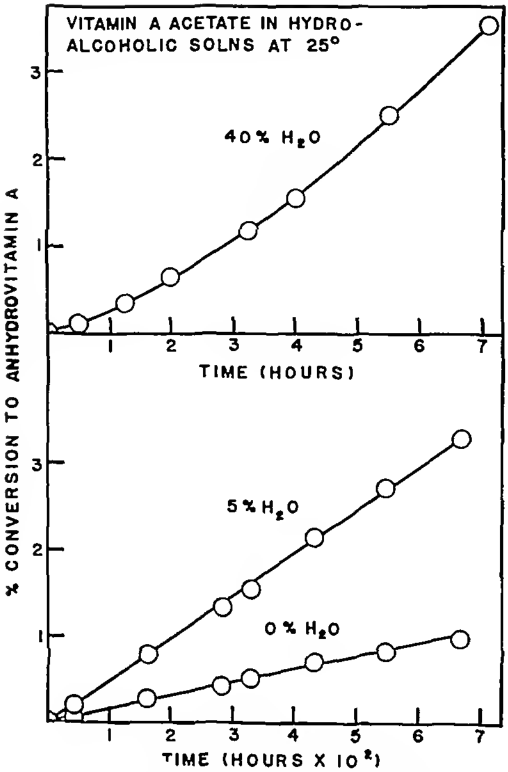


Fig 7—Rate of conversion of vitamin A acetate to anhydrovitamin A in ethanol with varying proportions of water at 25°

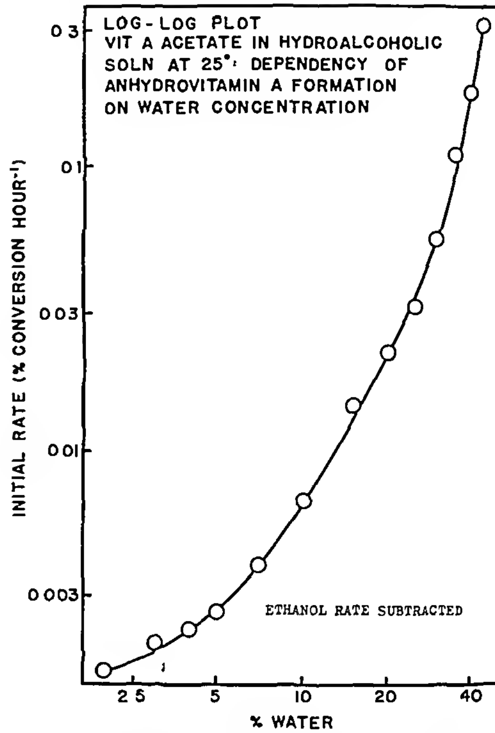


Fig 8—Variation in initial rate of anhydrovitamin A formation with concentration of water in ethanol at 25°

der reaches five at 45% water This very great dependence of the order on high water concentrations is difficult to explain One possibility is that there is a change in mechanism at the higher concentrations The data in Fig 7 lend some support to this At low concentrations of water in ethanol (0% and 5% are shown), the plot of per cent conversion *vs* time approximated a straight line, which would be expected for the first few per cent conversion But the plot for 40% water in ethanol had a positive second derivative Curves of this type as previously pointed out are shown by reactions which are complex

When the time axis of the latter curve was extended to 300 hours (Fig 3), the per cent conversion reached a peak at about 23% and then declined because of degradation of the anhydro compound From the low yield[1] it would appear that there are other products of the degradation, probably addition compounds, which are formed concomitantly [2]

Anhydrovitamin A Formation From Vitamin A Alcohol and Esters in the Presence of Various Additives

The effects of hydrogen chloride, perchloric acid, sodium hydroxide, and pyridine were studied because they were convenient for investigating acid and base effects which might give some insight into the general elimination reaction The effects of acetic acid and alpha tocopherol on the reaction were studied because substances of their type might be found in pharmaceutical formulations or appear on storage

Effect of Hydrogen Chloride on the Formation of Anhydrovitamin A—The qualitative effect of hydrogen chloride on the dehydration of vitamin A alcohol in ethanol was reported in the original work on anhydrovitamin A formation (1, 4) Hydrogen chloride also catalyzes the formation of the anhydro compound from vitamin A acetate, although the rate of the reaction with the alcohol is about 30 times faster than that with the ester This relationship is rather surprising in view of the fact that in ethanol, the alcohol form of the vitamin is nearly totally resistant to the elimination reaction, whereas the ester reacts at an appreciable rate

Catalysis by hydrogen chloride of anhydrovitamin A formation from vitamin A acetate in ethanol was studied at 25°, 30°, and 40° The reaction was found to be first order with respect to vitamin A acetate (Table I) and with respect to hydrogen chloride (Table II) The rate expression may be stated as

$$d\,(\text{Anhydro Vit A})/dt = k\,(\text{Vit A Acetate})\,(\text{HCl})$$

The values obtained for the specific reaction rate, k, are given in Table III An Arrhenius plot of logarithm of rate constant against reciprocal of the absolute temperature gave a heat of activation, ΔH, of 19 6 Kcal

To determine whether the principal catalytic species was *the proton or the hydrogen chloride* molecule, the rate of formation of anhydrovitamin A from vitamin A alcohol was studied in two other systems perchloric acid in 100% ethanol and hydrogen chloride in 40% water in ethanol

[1] Our studies of the acid catalyzed vitamin A alcohol and acetate elimination yielded from 30 to 60% conversion Robeson and Baxter (10) obtained 62% conversion

[2] All the reactions respons … vitamin A in these systems must be fir … lots indicate that the rate of overall … dependent on the substrate concentration

Although perchloric acid is intrinsically a stronger acid than hydrogen chloride (12), the rate of formation of anhydro A from vitamin A in the perchloric acid catalyzed system was less than half that in the hydrogen chloride catalyzed system. This seemed to indicate that the principal catalytic species was not some protonated acidic substances but the uncharged catalyst.

The data from the second system studied pointed to the same conclusion. When the reaction was run in 40% water in ethanol, the rate of formation of the anhydro compound from vitamin A was less than one-tenth the rate in pure ethanol. In the hydroalcoholic system the hydrogen chloride would be present almost entirely as oxonium and chloride ions, whereas in the pure ethanol system there would be relatively more hydrogen chloride present as the undissociated molecule. That the rate was greater in the pure ethanol system suggested strongly that the principal catalytic species was the hydrogen chloride molecule.

TABLE I.—DEPENDENCE OF HYDROGEN CHLORIDE CATALYZED ELIMINATION ON THE CONCENTRATION OF VITAMIN A ACETATE AT 25° (0.01 N HCL)

Concn of Vit A Acetate, mg /L	Initial Rate, mg liter^{-1} sec^{-1}
100	0 0048
200	0 0101
400	0 0200
800	0 044

TABLE II.—DEPENDENCE ON HYDROGEN CHLORIDE CONCENTRATION OF THE RATE OF ANHYDROVITAMIN A FORMATION FROM VITAMIN A ACETATE (60 MG. PER LITER)

Molar Concn HCl	Initial Rate, mg liter^{-1} sec.$^{-1}$ $\times 10^5$ 30°	40°
1×10^{-4}	5.4	14
2×10^{-4}	9 3	29
4×10^{-4}	20.0	62
6×10^{-4}		88
8×10^{-4}	46 0	124

TABLE III.—VITAMIN A ACETATE ELIMINATION CATALYZED BY HYDROGEN CHLORIDE

Temperature, ° C	Specific Reaction Rate[a] liters mole^{-1} sec^{-1}
25	$6\ 2 \times 10^{-3}$
30	$10\ 6 \times 10^{-3}$
40	$30\ 4 \times 10^{-3}$

[a] Calculated from initial rates of formation of anhydro A

Dependence of the Rate of Hydrogen Chloride Catalyzed Anhydrovitamin A Formation on Ethanol Concentration.—It was found that the rate of hydrogen chloride catalyzed elimination in nonhydroxylic solvents was very low In the absence of catalysts, as was stated earlier, the reaction was not detected. These studies, run at 0 01 N hydrogen chloride concentration for convenience, show the dependence of the hydrogen chloride catalyzed rate on the concentration of a hydroxylic solvent, ethanol. The systems studied were vitamin A alcohol in ethanol:heptane and in ethanol:isopropyl ether.

Limited solubility of hydrogen chloride in heptane prevented the use of low concentrations of ethanol in the ethanol:heptane experiments. A plot of logarithm of initial rate against logarithm of initial concentration is shown in Fig. 9. The slope of the curve is equal to the order of the reaction with respect to ethanol. In the concentration range studied (40–100% ethanol; the point at 20% ethanol is uncertain) the dehydration of vitamin A alcohol in the presence of hydrogen chloride was first order with respect to ethanol.

The dependence of the rate of dehydration on ethanol in the range 5–100% ethanol in isopropyl ether can be seen in Fig. 10. At the lower concentrations the slope of the curve indicated that the order approached two with respect to ethanol. As the concentration of ethanol was increased, the order gradually decreased until at high concentrations, the dehydration was first order with respect to ethanol.

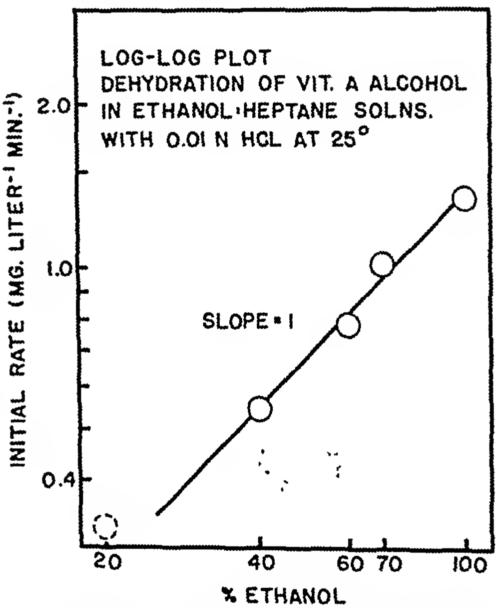


Fig 9.—Log-log plot of vitamin A alcohol in ethanol:heptane solutions with 0.01 N hydrogen chloride at 25°. Shows dependence on ethanol concentration of initial rate of formation of anhydrovitamin A from vitamin A alcohol.

Effect of Pyridine on the Rate of Formation of Anhydrovitamin A.—It was thought that if the mechanism of elimination for vitamin A acetate in ethanol were proton activated, then a proton scavenger would reduce the rate. The system studied to test this possibility was vitamin A acetate (60 mg. per liter) in ethanol at 90°, alone and with 0.001 N pyridine Pyridine was selected because it should tie up free protons without hydrolyzing the ester. The solutions with and without pyridine showed the same rate of formation of anhydrovitamin A. This seemed to indicate that the mechanism of formation of the anhydro compound from vitamin A acetate in ethanol was probably not exactly that suggested for proton catalyzed dehydration of vitamin A alcohol.

Effect of Sodium Hydroxide on Vitamin A Acetate in 40% Water in Ethanol.—To test the effect of strong base on the rate of anhydrovitamin A formation from vitamin A acetate, solutions were made

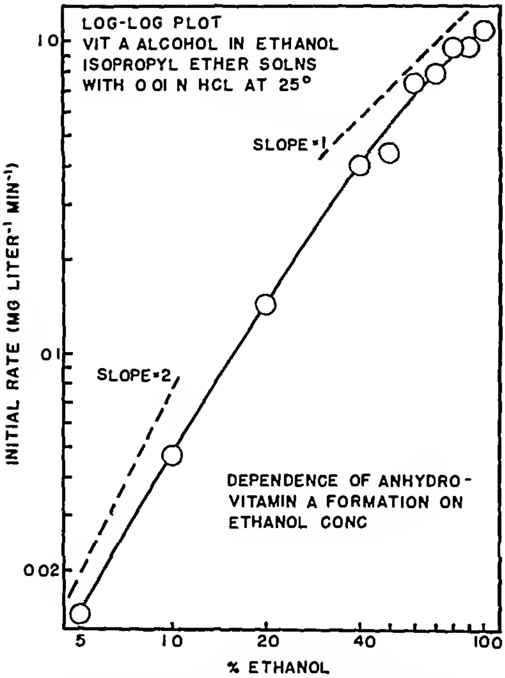


Fig 10—Log log plot of vitamin A alcohol in ethanol isopropyl ether solutions with 0 01 N hydrogen chloride at 25° Shows dependence on ethanol concentration of initial rate of formation of anhydrovitamin A from vitamin A alcohol

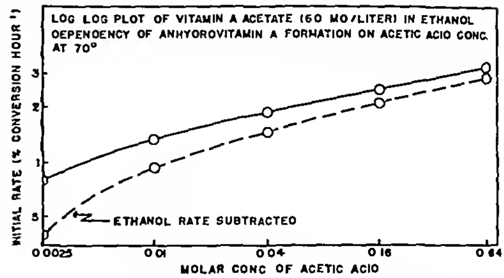


Fig 11—Log log plot of the dependence on acetic acid concentration of the rate of formation of anhydrovitamin A from vitamin A acetate in ethanol

with 60 mg per liter of vitamin A acetate and sodium hydroxide at 0 001 N and 0 02 N concentrations After fifty days at 25° these solutions showed very little change in their ultraviolet spectra

If the base hydrolyzed the ester very rapidly and left vitamin A alcohol in solution, this would explain the stability toward anhydrovitamin A formation, since we have shown that the alcohol form does not degrade at that concentration in ethanol at 25° in that period of time But in order for us not to detect any anhydro form, the hydrolysis would have had to occur within minutes, and this seemed very unlikely at room temperature Consequently, sodium hydroxide appeared to have stabilized the acetate against elimination This is contrary to what one might expect of base, since it usually catalyzes elimination reactions

Dependence on Acetic Acid Concentration of the Rate of Anhydrovitamin A Formation in Ethanol.—Dependence on acetic acid concentration of the rate of formation of anhydrovitamin A from the acetate in ethanol was studied at 70° over a 265 fold range The results are shown in Fig 11 The solid line represents the overall rate, the dashed line the difference between the observed rate and that of alcohol alone Over the range studied, the dependence of the rate on acetic acid appears to vary from first order at 0 0025 N acetic acid to one fourth at 0 64 N

The study showed that the presence of the acetic acid molecule increased the rate of conversion of vitamin A acetate to anhydrovitamin A significantly It seems, however, that the carboxylic acid catalyzed reaction would not be responsible for the major portion of the degradation of vitamin A ester, especially in hydroalcoholic solutions

Influence of α-Tocopherol on the Rate of Anhydrovitamin A Formation from Vitamin A Esters.—Phenolic antioxidants such as tocopherol are commonly used in vitamin A preparations to retard oxidative loss of the vitamin There is no question that it exerts this effect in oil solutions, but there seems to be some doubt as to its effect in aqueous dispersions (13, 14)

At 0 5% concentration α tocopherol was found to have no significant effect on the rate of formation of anhydrovitamin A from vitamin A acetate or palmitate As one may expect the acidity of this compound was apparently too weak to endow it with any catalytic activity for the elimination reaction

DISCUSSION

The exact nature of the mechanisms and pathways taken in the formation of anhydrovitamin A from either the acetate or the alcohol is impossible to delineate with presently available information It is clear that the transformation is not a simple reaction and in all probability is an end result of one of many competing mechanisms There also appears to be some evidence for formation of an intermediate species which then decomposes to form anhydrovitamin A In all likelihood addition, isomerization, elimination, and other reactions are occurring simultaneously

In the data collected, however, some semblance of order exists Vitamin A alcohol in ethanol would be expected to be stable to dehydration in the absence of catalysts, as was found If the catalysis by hydrogen chloride were by oxonium ion, the alcohol form would yield anhydrovitamin A faster than the acetate form because it could form a carbonium ion more easily From the evidence, it appears that oxonium ion is not the principal catalytic species, but the mechanism of elimination may be similar since the alcohol form is converted to the anhydro form more readily than is the acetate in the presence of hydrogen chloride

The acetate undergoes conversion to anhydrovitamin A in the presence of hydroxylic solvents and acids For this compound, such solvents appear to be necessary for rapid reaction even in the presence of strong catalysts At first glance it might seem that the elimination reaction by which vitamin A acetate is converted to the anhydro form follows a straight E_2 pathway, with the ethanol or water solvent acting as the base If this were true, a stronger

base such as sodium hydroxide would be expected to speed up the reaction. Since it appears that sodium hydroxide is actually an inhibitor, the reaction is probably not through a simple E_2 mechanism.

PHARMACEUTICAL SIGNIFICANCE

Deterioration of vitamin A esters in pharmaceutical preparations by conversion to the anhydro form does not seem to have received much attention in the literature. Since the reaction can take place in the absence of strong catalysts in hydroxylic solvents, detection of anhydrovitamin A would appear to be a necessary part of studies on the degradation of vitamin A in solution or in dispersion in these solvents. Some studies reported up to now (13, 14) have measured only the absorption at 326 mμ, or at 620 mμ in the Carr-Price procedure, neither of which would show the presence of the anhydro form. The Carr-Price method, in particular, is not suitable for systems which form the anhydro species since the latter gives a blue color very similar in wavelength and intensity to that of vitamin A itself.

EXPERIMENTAL

Reagents.—Crystalline vitamin A alcohol, vitamin A acetate, and vitamin A palmitate (Hoffmann-La Roche); α-tocopherol (Hoffmann-La Roche); pyridine, perchloric acid, hydrochloric acid, acetic acid, sodium hydroxide—all reagent grade; *n*-heptane (Eastman, white label)—shaken with sulfuric acid, then passed through Davison silica gel (through 200 mesh); isopropyl ether (Matheson Coleman and Bell, Pract.)—peroxides removed by treatment with acidified ferrous sulfate, dried with Drierite and distilled; "absolute" alcohol, N. F.—reacted with magnesium activated by iodine and then distilled using a 10:1 reflux distill ratio on a 50 plate Oldershaw fractionating column; water content after distillation: 0.014% (Karl Fischer titration); and double distilled water.

Apparatus.—Cary Recording Spectrophotometer Model 11MS with constant temperature jacket; constant temperature baths at 30°, 40°, 70°, and 90° ± 0.1°, and constant temperature room at 25° ± 0.5°, 10 ml. Neutraglas Color-Break Ampuls.

Procedure.—All studies were made in the absence of light. For reactions with half lives longer than about one hour, the formation of anhydrovitamin A was followed spectrophotometrically by withdrawing aliquots (for lower temperatures) or ampuls (for higher temperatures) at suitable intervals and measuring the absorbance at 392 mμ.

For reactions with half lives of the order of minutes, the appearance of the anhydro form was followed directly in a Cary spectrophotometer which had a water jacketed cell compartment to keep the temperature constant. A measured volume of substrate solution was pipetted into a silica sample cell. Then a measured volume of catalyst was pipetted into the cell, the solution shaken, placed in the sample compartment and the spectrophotometer, which was set to record at the appropriate wavelength, was switched on. The time elapsed between pipetting the catalyst into the cell and starting the recording was twelve seconds. The curve of concentration of anhydrovitamin A against time was obtained automatically.

The initial rate of formation of anhydrovitamin A was calculated from the rate of increase in absorbance at 392 mμ by the formula:

$$\text{Initial Rate of Formation of Anhydrovitamin A } (\text{mg liter}^{-1}\text{ min.}^{-1}) = \frac{\text{Initial Increase in Absorbance per min. at 392 m}\mu}{A^{1\%}_{1\text{ cm}} \times 10^{-4}} = \frac{\text{Initial Increase in Absorbance per min. at 392 m}\mu}{3180 \times 10^{-4}}$$

CONCLUSIONS

1. The formation of anhydrovitamin A was first order with respect to vitamin A.

2. Water caused a marked increase in the rate of formation of anhydrovitamin A in hydroalcoholic solutions. The order of the reaction with respect to water varied from less than one at 2 per cent water to five at 45 per cent water.

3. Catalysis by hydrogen chloride of anhydrovitamin A formation from vitamin A acetate was first order with respect to vitamin A and with respect to hydrogen chloride. The principal catalytic species appeared to be the whole hydrogen chloride molecule rather than the hydrogen ion.

4. At concentrations above about 50 per cent ethanol, the hydrogen chloride catalyzed dehydration of vitamin A alcohol in ethanol:heptane and ethanol:isopropyl ether solutions was first order with respect to ethanol.

5. Pyridine has no effect on the rate of anhydrovitamin A formation.

6. Sodium hydroxide appeared to inhibit the formation of anhydrovitamin A in solutions of vitamin A acetate in 40 per cent water:ethanol.

7. Acetic acid caused a distinct but relatively slight increase in the rate of anhydrovitamin A formation from vitamin A acetate.

8. α-Tocopherol had no effect on the rate of anhydrovitamin A formation.

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A Chemical Study of American Tung Oil*

By YONG CHOO LEE† and W. LEWIS NOBLES

A phytochemical investigation of American tung oil which had been reported to have hypotensive properties was carried out in an attempt to isolate the active component in a pure state. 9,14-Dihydroxy-10,12-octadecadienoic acid which had been reported previously in tung oil, was isolated by using Permutit A (anion exchange resin) rather than Ionac A. A white crystalline substance, m. p. 295–297°, was obtained in low yield from the 80 per cent alcohol extract of a petroleum ether solution of tung oil. It is a substance not previously reported in tung oil and appears to be a phytosterolin (phytosterol glycoside) according to its physical and chemical properties. From the fraction not containing eleostearic acid after hydrolysis, a syrupy extract was obtained by utilizing freeze-drying; from the saponifiable fraction, eleostearic acid was isolated.

The tung oil tree is *Aleurites fordii* Hemsley, belonging to the *Euphorbiaceae*. Various steps must be taken after the nuts are harvested to obtain tung oil. These include drying in the orchards, hulling, and expression or solvent extraction of the oil. Many factors, including the degree of moisture content, type of hulling, temperature, and nature of solvent affect the quantity and quality of the oil obtained.

Tung oil plays an important role in the coating industry, but it is not used in significant amounts in the medicinal field in this country at this time. In China, it is given (1) as a remedy in insanity and in the case of metallic poisoning; also it is applied as a stimulant to carbuncles, ulcers, burns, swellings, and bruises. A refined tung oil is currently being advertised in this geographical area (southeastern United States) for promoting the healing of cuts, wounds, and abrasions.

Kaufmann (2) has reported that tung oil contains 78.5–87.1% eleostearine, 7.6–22.8% olein, and 8.5% glycerides of saturated acids. Smedley-MacLean (3) has reported that tung oil is composed entirely of C_{18} fatty acids. Recently, Hoffmann and O'Connor (4) succeeded in isolating the *alpha*- and *beta*-eleostearic acids from tung oil using ethanol as the solvent for recrystallization.

Henry and Auld (5) suggested that there may be a cyanogenetic glycoside in the fruit of tung tree and that the poisonous nature of the press cake may be due to hydrogen cyanide produced by the hydrolysis of the glycoside during purification.

Emmel (6) determined the presence of two toxic principles in the tung nut. He indicated that the first toxic principle is probably a saponin and is in the foliage, sap, and fruit. He found that the second toxic principle in tung meal is partially destroyed by heat or hydrolysis and is present in greater quantity in fresh tung meals than that in the old meals.

Carratolá (7) stated that the liquid obtained by the incision of the fruit exerts a local irritation on the skin. He found that the liquid contains a substance like a toxalbumin, a natural albuminoid which acts similarly to strychnine in the human body. He did not isolate any alkaloids from this liquid. A solution of the substance, which was heated to 90° and injected into guinea pigs, elicited no toxic response.

Bilger and Westgate (8) reported the presence of sterols in tung oil and compared them with sterols obtained from other tropical oils. They found that tung oil possessed the following properties: unsaponifiable material, 0.59%; fraction precipitated by digitonin from an alcoholic extract, 37.30%; sterol in oil, 0.22%; molecular weight of the tung sterol, 365.

In the present study, we were particularly interested in the hypotensive factor reported in tung oil by Grollman (9). He suggested that the tung oil is apparently the only oil of vegetable origin which contains a factor of this type. Also, Karnovsky (10) reported that the only vegetable fat containing any appreciable quantity of alpha-glyceryl ethers is tung oil. However, whether or not there is any relationship between these two isolated bits of information remains yet to be determined.

Davis, Conroy, and Shakespeare (11) isolated a crystalline substance in low yield from tung oil and it was shown to be 9,14-dihydroxy-10,12-octadecadienoic acid, $CH_3(CH_2)_3CHOH—CH=CH—CH=CH—CHOH—(CH_2)_7COOH$, suggested as being an artifact derived from eleostearic

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acid. The hypotensive activity of this acid, reported by Grollman (9), was not confirmed by these workers.

This study was designed to attempt the separation, isolation, and identification of certain of the chemical constituents of tung oil. Particular emphasis was placed on those constituents possessing potential medical value. Specifically, attempts were made to isolate the hypotensive factor reported by Grollman (9) in larger quantities than ever before, identify it and, if possible, synthesize it.

EXPERIMENTAL

The following three methods were utilized for studying the tung oil fractions: ion exchange resin method; petroleum ether—ethyl alcohol method; and hydrolysis method.

Ion Exchange Resin Method

As ion exchange resins, cation exchangers (IR-100, IR-120)[1] and anion exchangers (Dowex 3[2], Permutit A[3]) were used. Taking into consideration that the chemical properties would be different according to the method of obtaining the oil, different kinds of oil were treated with each kind of resin.

Regeneration of Resins.—IR-120.—The resin was thoroughly washed with distilled water, conditioned with three cycles of regeneration with C. P. hydrochloric acid and exhaustion with 20% sodium hydroxide, and finally regenerated with a very large excess of analytical reagent grade hydrochloric acid, washed free of acid with distilled water, and air-dried.

Dowex 3.—A 250-Gm. portion of resin was stirred with 2.5 L. of 5% sodium carbonate for one-half hour, filtered, washed thoroughly with distilled water, and air-dried.

Permutit A.—A 250-Gm. portion of resin was stirred with 2.5 L. of 5% sodium hydroxide for one and one-half hours, filtered, washed thoroughly with distilled water, and air-dried. The reaction of the washings approached neutrality (pH 8.3).

General Procedure.—The method utilized was essentially that described by Davis, *et al.* (11), with the exception that Permutit A was used as the anion exchange resin in lieu of Ionac A. The results were analogous to those reported by the previous workers.

One result, which is unexplainable at the moment, is the failure of Dowex 3 to remove the acidic substances from the oil as did Permutit A.

The results of the use of both the cationic and the anionic resins are summarized in Table I.

Petroleum Ether-Ethyl Alcohol Method

a. A 750-Gm. portion of oil was dissolved in 750 ml. of petroleum ether in a 3-L. separatory funnel and shaken three times with 300 ml. of 80% ethyl alcohol. When the amount of petroleum ether was insufficient, an emulsion occurred on shaking with the alcohol. The alcohol layer was yellow and transparent. An additional 2,600 Gm. of the oil was treated in the same manner. The combined alcoholic extract was allowed to stand in an evaporating dish at room temperature. As the alcohol evaporated, a colorless needle-like crystalline substance was produced on the resinous surface of the liquid. A sample of this crystalline substance was removed; it melted at 40° and became resinous in the air.

The alcoholic extract was completely resinified after a month; it was treated with small amounts of cold alcohol several times. Most of the resin dissolved in the alcohol and a mixture of a white granular material and a colored film remained. The residue was washed with hot ethyl acetate and treated with hot ethyl alcohol several times. Only the colored film (about 0.1 Gm.) remained; it was removed by filtration. From the combined alcoholic filtrate, a white substance, m. p. 265–270°, was obtained by evaporation of the solvent. This was twice recrystallized from 95% ethyl alcohol with the aid of animal charcoal, yielding a white, needle-like crystalline substance, m. p. 295–297°.

On standing at room temperature for a long time, the same crystalline substance was obtained from the alcoholic filtrate of the original extract, from which the mixture of the white granular material and the colored film had been separated. The total yield was 265.1 mg. (0.0071%).

This crystalline substance was quite insoluble in water, 10% sodium hydroxide, and 10% hydrochloric acid. It was very sparingly soluble in alcohol, methanol, ethyl acetate, ether, acetone, benzene, carbon tetrachloride, and petroleum ether; readily soluble in pyridine, dioxane, and amyl alcohol. Its alcoholic solution was not colored with aqueous ferric chloride. Most of the sterol color tests (Salkowski test, Liebermann-Burchard test, and Tschugajeff test) were positive, but the Rosenheim test was negative. It was immediately colored dark red on treatment with concentrated sulfuric acid. Analysis yielded the following results:

Anal.—C, 72.94, 72.67; H, 10.55, 10.39.

Hydrolysis.—The method utilized for this process was that of Power and Salway (12). Eighty-nine milligrams of the substance (m. p. 295–297°) was dissolved in 5.4 ml. of hot *n*-amyl alcohol; 1.8 ml. of an aqueous 15% solution of hydrogen chloride was added together with sufficient ethyl alcohol (7 ml.) to form a homogeneous liquid. After heating for one hour in a reflux apparatus, steam was passed through the mixture to remove the amyl alcohol. A colored solid was produced, removed by filtration, and washed with distilled water. When the solid substance was crystallized from a mixture of ethyl alcohol and ethyl acetate (1:1 by volume), it occurred as glistening leaflets (15 mg.) melting at 133°. This substance gave positive Liebermann-Burchard and Tschugajeff color tests, but was negative in the Rosenheim test. Carbon and hydrogen analyses yielded the following:

Anal.—C, 80.24, 80.49, H, 11.65, 11.43.

The aqueous acid liquid, from which the phytosterol had been separated by filtration, was neutralized with sodium carbonate and evaporated to dryness on the water bath. The residue was digested with absolute ethyl alcohol several times and the mixture filtered. On evaporating the alcoholic

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[1] IR-100 and IR-120, products of Rohm & Haas Co., Philadelphia 5, Pa.
[2] Dowex 3, a product of Dow Chemical Co., Midland, Mich.
[3] Permutit A, a product of Permutit Co., 330 W. 42nd St., New York 36, N. Y.

TABLE I —CATION AND ANION RESIN EXTRACTS FROM TUNG OIL

Experiment No	Type of Resin	Resin	Oil Gm	Extract Gm	Acid mg
1	Cation	IR 100	3367	0 015	
2	Cation	IR 100	1272	0 0081	
3	Cation	IR 120	1464	0 0037	
4	Cation	IR 120	7213	0 01	
5	Cation	IR 120	7397	0 027	
6	Cation	IR 120	17099	0 065	
7	Cation	IR 120 Regenerated	11320	0 0403	
8	Anion	Regenerated Dowex 3	2500	0 0096	
9	Anion	Regenerated Permutit A	12003	8	210 7

trate, a small amount of a syrupy residue was obtained This had a caramel odor, reduced Fehling's solution and gave a positive Molisch reaction, but no osazone was obtained

b A 14,010 Gm portion of a solvent extracted tung oil was treated according to the general procedure outlined previously The previous oil sample had been obtained by mechanical expression The yield of the crystalline substance m p 295–297° was, however, only 6 mg (0 00005%) The yield of the 80% ethyl alcohol extract was about 90 Gm This was reserved for pharmacological evaluation

Hydrolysis Method

A 200 Gm portion of a tung oil was saponified by refluxing in a 1 L round bottom flask with 60 Gm of potassium hydroxide, 50 ml of water, and 500 ml of 95% ethyl alcohol according to the previously described method (4) The flask and its contents were swirled occasionally during the thirty minute saponification period After cooling, the soap was acidified in a separatory funnel with 725 ml of 2 *N* hydrochloric acid The liberated acids were separated from the aqueous layer and dissolved without further treatment in 1 L of 95% ethyl alcohol on the water bath The solution stood for twenty four hours in the refrigerator The light colored precipitate was removed by filtration, then washed with about 75 ml of cold 95% ethyl alcohol In order to avoid air oxidation of this substance during filtration, the rate of filtration was adjusted so that solvent vapors were always in contact with the crystals It was recrystallized from 500 ml of 95% ethyl alcohol, the yield was 75 Gm, m p 48°

The residual yellow aqueous phase, which possibly could contain some water soluble acidic substances, was neutralized with 20% potassium hydroxide, and evaporated at room temperature When most of the alcohol had evaporated, the volume of the solution was 950 ml, the solution was yellow and possessed a characteristic odor This was frozen with dry ice and acetone, and by means of the Campbell Pressman Apparatus [4] was subjected *to freeze drying* One hundred twenty nine grams of light colored solid was obtained Most of this was inorganic in nature It was treated twice with 100 ml of cold absolute ethyl alcohol and the combined filtrate was evaporated at room temperature A yellow syrupy extract (16 Gm) was obtained This was reserved for pharmacological evaluation

[4] This apparatus is produced by F Machlett & Son Co 220 East 23rd St, New York 10 N Y

DISCUSSION OF RESULTS

Ion Exchange Resin Method.—As may be noted in Table I, in most of the cases, a small amount of extract was obtained except with Permutit A In the case of Permutit A, a considerable quantity of extract and a small amount of crystals were obtained It was established that this crystalline material was identical with the 9,14 dihydroxy-10,12 octadecadienoic acid which Davis, *et al* (11), had isolated from tung oil by the use of an anion exchange resin (Ionac A300)

If this acid had hypotensive activity and is an artifact resulting from autoxidation of eleostearic acid during production and fractionation, as Davis, *et al* (11), have suggested, conditions for the autoxidation of eleostearic acid in the experiment should be studied for the purpose of obtaining a better yield Comparison of yield Davis, *et al*, 0 0027% (crystals of m p 93–98°), present investigation, 0 0018% (m p 102–103°)

Petroleum Ether-Ethyl Alcohol Method.—The melting point of the white crystalline substance, m p 295–297°, is considerably higher than those of the sterols which were reported by Bilger, *et al* (8) Indeed, the melting points of the ordinary free phytosterols are mostly less than 200°, ergosterol m p 113°, stigmasterol m p 169°, fucosterol m p 124°, etc

Some sterol color tests also give positive results with saponins This substance, however, is different from the saponin which was reported by Emmel (6) for the following reasons Most of the saponins are soluble in water and elicit characteristic foaming but this substance does not, and generally, saponins are amorphous with the exception of diosein, sarsasaponin, and cyclamin, but this substance is in the form of microscopical needles

Elemental analysis of this substance indicated that no nitrogen is present Accordingly, it is also different from the cyanogenetic glycoside reported by Henry and Auld (5) and the albuminoid reported by Carratola (7)

It thus appears that this substance is a constituent not previously reported in tung oil

Presumably, this substance belongs to the phytosterolins (phytosterol glycosides) because of the following properties (*a*) It elicits a positive sterol color test (*b*) Its elemental analysis is very similar to that of phytosterolins (12) (*c*) On hydrolysis, this substance yielded a crystalline material, m p 133°, which gave a positive sterol color test, and a syrupy substance which reduced Fehling's solution and gave

a positive Molisch reaction. (*d*) It exists as microscopically small needles and its melting point is relatively high and not sharp. These properties are common in phytosterolins (*e*) Hall and Gisvold (13) obtained simultaneously a free sterol and its glycoside from the same plant By analogy, it is probable that the glycosides of the free sterols reported by Bilger, *et al* (8), exist in the tung oil. (*f*) The process of isolation of it is similar to that of phytosterolins Most of the phytosterolins were isolated from an organic solvent extract, sometimes they result from aqueous extracts by preserving them for a long period of time

The percentage content of this crystalline substance in various types of tung oil is apparently different The percentage yield of this substance in different experiments was 0 0071% and 0 00005%, respectively The amount of this substance obtained from tung oil by the expression method may be more than that obtained by the solvent method, as this substance is quite insoluble in ordinary organic solvents

The crystalline material, m p 40°, may be impure eleostearic acid which was produced during the processing of tung oil, since this crystalline substance resinifies in the air Eleostearic acids generally give this characteristic behavior.

Hydrolysis Method.—Originally, this method was designed to study the fraction of tung oil not containing eleostearic acid The freeze-drying techniques were utilized to avoid the decomposition of any heat labile component Further chemical treatments of the yellow syrupy extract are in progress

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A Study of LSD-Serotonin Central Interaction*

By PAUL ZANOWIAK† and MORTON J. RODMAN‡

Serotonin pretreatment increases the duration of sleep produced by a variety of central depressants. Lysergic acid diethylamide (LSD) pretreatment inhibits or completely reverses such serotonin potentiation of sleep produced by hexobarbital, mephenesin, and combinations of depressants in which these drugs exert predominant actions. LSD does not suppress serotonin potentiation of hypnosis produced by butabarbital and meprobamate; on the contrary, LSD alone increases the duration of sleep with these compounds to about the same degree that serotonin does.

It has been suggested that serotonin plays a part in both normal and abnormal mental processes and that the striking psychological effects of lysergic acid diethylamide (LSD) are the result of its ability to interfere with some central action of serotonin (1, 2).

The hypotheses are based in part on the occurrence of serotonin in the brain and the fact that LSD specifically antagonizes the actions of serotonin at a number of peripheral sites *in vitro* (3, 4). More recently, several pharmacological tests have been proposed for demonstrating a similar central antagonism (5, 6, 7).

One such method of evaluating central antiserotonin action is based upon the finding that serotonin prolongs barbiturate hypnosis and that this action is suppressed by prior administration of certain serotonin antagonists. Those properties of serotonin and LSD have been demonstrated in mice, rats, and rabbits depressed by hexobarbital, thiopental, and thialbarbitone (7, 8, 9).

The purpose of this study was to determine whether the results reported in animals receiving such thiobarbiturates could be reproduced when hypnosis was brought about by certain other types of central depressants, administered alone or in various combinations.

METHODS AND MATERIALS

Unfasted male Swiss albino mice from 18–25 Gm were used only once Animals were obtained from a single source and kept in the laboratory for at least two days prior to use, to avoid variation. All experiments were carried out during a four-week

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period in the spring of a single year. Room temperature was controlled at approximately 70° F. Thus, differences in sleeping time are not attributable to variation in the environmental temperature.

Control groups were used in all experiments, each animal receiving an injection of saline solution equivalent to the volume of drug solutions employed. The criterion for duration of sleeping time was the time interval between loss and return of the righting reflex. Due to difficulty in determining a firm end point under some circumstances, animals were returned to their backs after apparently spontaneously righting and this was considered a true return of the reflex only when the animal righted itself again within thirty seconds. Standard errors of the mean sleeping times were calculated by the method of Miller and Tainter (10).

All drugs were administered intraperitoneally in the form of freshly prepared solutions. LSD was given in a dose of 10 mg /Kg. one hour before the depressants. Serotonin in a dose of 20 mg./Kg. as serotonin creatinine sulfate, was injected ten minutes before the hypnotics. The depressants were administered in doses which produced a standard degree of central depression, the MED_{50}. This was the dose required to produce hypnosis for a minimum specified period (thirty or sixty minutes) in half of the group of animals. For hexobarbital, and the lower dose levels of mephenesin and meprobamate, the MED_{50} is for thirty minutes; for butabarbital and the larger doses of mephenesin and meprobamate, the MED_{50} is for sixty minutes. The dosages for the combinations consisted of one-half MED_{50} of each barbiturate combined with mephenesin and meprobamate in amounts known to act synergistically with the barbiturates to produce an effect equal to that of one MED_{50} of each barbiturate.

All these doses were derived from an investigation of barbiturate-lissive synergism conducted previously in this laboratory in accordance with the procedure of Gaddum (11), as employed by Berger and Lynes (12). As apparent from an inspection of the various doses in the tables: hexobarbital and mephenesin were additive, butabarbital and mephenesin were incompletely additive, and meprobamate potentiated the depressant action of both barbiturates.

RESULTS

A characteristic sequence of behavioral changes was observed in all animals receiving LSD. The mice became agitated shortly after injection. This was manifested by continual movements of the head from side to side. Often the animals circled rapidly and restlessly about the cage. On other occasions this pattern, which was suggestive of searching, was replaced by one of apparent fear. The animals walked backward in an oddly uncoordinated manner, and, when touched, they would react with strong almost convulsive escape activity. Bristling of the hair and reddening of the ears also occurred frequently. Such excitement began to diminish about thirty minutes afterward and had ceased entirely at the end of one hour, just prior to administration of the depressant. Frequently, the animals would now lie quietly in one position and offer little resistance to the subsequently administered injections.

Serotonin administered by itself ten minutes before the depressant did not produce loss of the righting reflex. It did, however, consistently cause a decrease in spontaneous activity. During this period of slight depression, the mice would lie quietly in one position but were capable of moving when stimulated.

The results of administration of the drugs alone and in various combinations are summarized in Tables I, II, and III. The values stated refer to the mean sleeping times in minutes and the standard error. The figures in parentheses indicate the number of animals used in each series.

The results obtained with hexobarbital, LSD, and serotonin are essentially in accordance with those previously reported in experiments of this type; that is, serotonin potentiated hypnosis produced by the barbiturate and this action was partially suppressed by prior administration of LSD. LSD alone considerably reduced hexobarbital hypnosis, contrary to one reported observation indicating that LSD by itself had no effect on barbiturate sleeping time (13).

The results of the two mephenesin series were likewise in accordance with the results reported previously for barbiturates, except that LSD completely counteracted serotonin potentiation of mephenesin hypnosis, reducing the duration of sleep to a point somewhat below that attained with mephenesin alone at both dose levels.

With butabarbital and meprobamate, on the contrary, the responses to LSD alone and to LSD combinations were quite different from those reported for thiobarbiturates and those observed here with mephenesin. Pretreatment with LSD prolongs the duration of hypnosis produced by these agents in a manner indistinguishable from the potentiating effect of serotonin. Likewise, pretreatment with LSD does not significantly inhibit such serotonin potentiation of the central depressant action of these compounds. This is especially true in the case of butabarbital, in which potentiation by LSD alone, serotonin alone, and combined LSD-serotonin is strikingly similar; with meprobamate some slight suppression of serotonin potentiation is apparent, but the duration of sleep is still nearly double that of the control group at the lower dose level.

Responses of the animals receiving combinations of depressants after pretreatment with LSD and serotonin alone and in combination were comparatively variable. However, the effects on duration of hypnosis appeared, generally, to be related to those which might be predicted in the light of the foregoing findings. That is, potentiation, and suppression of this response appeared to depend largely on the type of depressants employed to produce the basic narcosis. Thus, for example, in animals treated with the hexobarbital-mephenesin combination, the effects after pretreatment were similar to those which had occurred when each of the depressants had been used separately.

With butabarbital-meprobamate, which like hexobarbital-mephenesin is a combination of drugs producing essentially similar responses, the results were also largely as might have been predicted. Failure of serotonin to potentiate hypnosis significantly in this series is a notable exception.

TABLE I.—EFFECTS OF PRETREATMENT OF MICE WITH LSD AND SEROTONIN ON LENGTH OF HYPNOSIS OF RELATIVELY SHORT DURATION

Drug and Dose, mg./Kg.	Depressant Alone, minutes	Depressant plus LSD, minutes	Depressant plus Serotonin, minutes	Depressant plus LSD Plus Serotonin, minutes
Hexobarbital 100	28.0 ± 1.3 (50)[a]	17.3 ± 1.0 (10)	53.2 ± 1.4 (19)	38.0 ± 2.4 (10)
Mephenesin 320	28.0 ± 1.2 (43)	16.3 ± 1.0 (10)	42.1 ± 1.9 (20)	24.4 ± 1.3 (10)
Meprobamate 240	44.9 ± 2.3 (30)	84.7 ± 5.3 (10)	97.5 ± 1.8 (20)	86.6 ± 1.8 (10)

[a] The figures in parentheses indicate the number of animals used.

TABLE II.—EFFECTS OF PRETREATMENT OF MICE WITH LSD AND SEROTONIN ON LENGTH OF HYPNOSIS OF MODERATELY LONG DURATION

Drug and Dose, mg./Kg.	Depressant Alone, minutes	Depressant plus LSD, minutes	Depressant plus Serotonin, minutes	Depressant plus LSD plus Serotonin, minutes
Butabarbital 56	75.8 ± 2.7 (60)[a]	153.0 ± 7.7 (20)	146.0 ± 5.5 (19)	154.9 ± 7.7 (10)
Meprobamate 280	70.8 ± 4.4 (38)	131.0 ± 7.5 (10)	127.6 ± 4.0 (18)	107.0 ± 3.6 (10)
Mephenesin 435	76.7 ± 1.1 (30)	45.1 ± 1.4 (10)	125.6 ± 1.7 (20)	46.9 ± 3.6 (10)

[a] The figures in parentheses indicate the number of animals used.

TABLE III.—EFFECTS OF PRETREATMENT OF MICE WITH LSD AND SEROTONIN ON LENGTH OF HYPNOSIS PRODUCED BY COMBINED DEPRESSANTS

Drug Combination and Doses, mg./Kg.	Depressant Alone, minutes	Depressant plus LSD, minutes	Depressant plus Serotonin, minutes	Depressant plus LSD plus Serotonin, minutes
Hexobarbital 50; Mephenesin 160	27.8 ± 1.5 (30)[a]	23.2 ± 1.0 (10)	64.0 ± 3.7 (20)	27.0 ± 1.9 (10)
Hexobarbital 50; Meprobamate 72	22.3 ± 0.9 (30)	34.3 ± 2.6 (10)	34.6 ± 3.3 (20)	25.0 ± 1.6 (10)
Butabarbital 28; Mephenesin 300	62.6 ± 2.2 (30)	53.7 ± 2.9 (20)	158.0 ± 5.6 (20)	82.6 ± 4.2 (10)
Butabarbital 28; Meprobamate 67	58.9 ± 1.1 (30)	91.2 ± 3.7 (10)	65.4 ± 2.7 (20)	118.0 ± 7.5 (10)

[a] The figures in parentheses indicate the number of animals used.

In the series in which combinations of differently acting depressants were used, the data are most variable. However, this could be expected, due to the different responses elicited by each drug administered alone after LSD and serotonin pretreatments. Generally, the duration of sleeping time tended to be influenced by the predominant drug in each mixture. Thus, for example, the partial suppression by LSD of serotonin potentiation of hypnosis in both series may be due to the predominance in each case of the drug, which, when used alone, elicits a similar response. In the hexobarbital-meprobamate combination, this is the barbiturate; in the butabarbital-mephenesin combination, the effects of the latter drug predominate. In each combination, the drug exerting the more influential effect is the one which is present as a greater fraction of its MED_{50}, and which, administered alone, would cause a longer depression.

DISCUSSION

Despite a paucity of direct evidence that serotonin takes part in normal brain function at all, it has been suggested that an abnormality in central serotonin function may be the basis of certain mental disturbances (14–16). According to these views, mental illness may be the result of a metabolic function resulting in the development of either a functional deficiency or excess of serotonin at the central sites at which it performs certain postulated physiological functions.

Because of the remarkable potency of LSD in producing symptoms similar to some seen in schizophrenia and its antagonism of certain peripheral effects of serotonin this synthetic ergot alkaloid and its congeners have been widely employed as pharmacological tools in the investigation of these various working hypotheses (6, 7, 17). Data obtained in such studies, while often meager and indirect, have been interpreted in terms that favor one or another of the current biochemical concepts of mental disorder.

While we are unwilling to attempt an interpretation of our own equally indirect results in terms of the complex problem of whether serotonin plays a part in normal and abnormal mental processes, it may be profitable to consider the data in relation to the more limited problem of whether the psychological effects of LSD are related to a central interaction with serotonin, as has been suggested.

Data, such as those reported here for hexobarbital-serotonin-LSD interactions, have been offered as evidence for the concept that LSD suppressed some central action of serotonin (7). It has been suggested that such suppression of serotonin potentiation of hexabarbital hypnosis is the result of replacement of serotonin and its action at receptors by LSD (13). The fact that LSD by itself had little effect on hypnosis produced by the barbiturate has been explained in terms of the antimetabolite concept. That is, LSD, a structural analog of serotonin, is believed to act as an antiserotonin, because it is sufficiently close to serotonin in chemical configuration to combine with its receptors without,

however, being able to fulfill the physiological role of the hormone (1, 5, 15, 16)

However, this concept of a central antagonism between LSD and serotonin is not borne out by the results of our experiments, in which butabarbital and meprobamate, rather than hexobarbital, are the basic depressants Here, not only does LSD fail to reverse serotonin potentiation of hypnosis, but LSD by itself prolongs the duration of hypnosis to an extent at least equal to the effect of serotonin alone on the action of these depressants Thus, in these experiments, LSD appears to produce a "serotoninlike" response This may mean that LSD acts, not by creating a functional deficiency of serotonin, but an excess

The view that LSD may mimic the action of serotonin rather than compete with it is supported by a number of other observations in which LSD had an effect similar to that of serotonin (18) Depending upon the test object and concentration of LSD employed, both antiserotonin and serotoninlike results have been reported Wooley and Shaw (18) have attempted to account for these actions by suggesting that serotonin receptors are not all alike but vary from tissue to tissue LSD may then be pictured as fitting some of these receptors well enough, not only to block the effects of serotonin, but to itself induce a serotoninlike effect

Our data especially for the butabarbital series, offer some support for this concept of LSD combining with serotonin receptors well enough to act like the hormone itself There is little difference in the degree to which LSD alone and serotonin alone potentiate butabarbital hypnosis Furthermore, pretreatment with both LSD and serotonin produces a potentiation of butabarbital hypnosis that differs very little from that caused by pretreatment with either substance alone This failure of serotonin to increase duration of butabarbital sleep beyond that brought about by LSD alone may mean that the hormone cannot combine with receptors already occupied by LSD

While the antimetabolite theory may be invoked to account for both the antiserotonin and serotoninlike actions of LSD in these experiments, one crucial question remains unanswered What properties of the drugs themselves account for the differences in their interactions with LSD and serotonin?

One property which must be considered is the difference in duration of action of these depressants Because butabarbital and meprobamate are classified as relatively long in action compared to hexobarbital and mephenesin, it might be argued that the difference in response to the two groups after LSD pretreatment may be due to a temporal factor Thus, for example, early LSD stimulation might be followed by a late depressant action which would synergize with that of the longer acting drugs, with the short acting compounds, on the other hand, recovery could occur before this delayed depressant action of LSD developed

That such a temporal relationship is not involved, however, is indicated by the results of a number of experiments where LSD and the barbiturates were administered simultaneously The effects of LSD on the duration of hypnosis were nearly identical with those produced when it was given one hour prior to the barbiturate, hexobarbital sleeping time was reduced and butabarbital hypnosis nearly doubled

Evidence that the differences in the interactions of the depressants with LSD are independent of the duration of action of the drugs is also seen in the results of the mephenesin series Mephenesin, whether employed in doses that cause sleep of long or short duration, produces a characteristic response quite different from that of butabarbital and meprobamate, when all three drugs are given in equipotent doses in animals pretreated with LSD and serotonin (Table II)

Differences in interactions of different drugs with LSD and serotonin might also be accounted for in terms of their varying sites and mechanisms of action Thus, the response to a drug acting at cells possessing one type of serotonin receptor might well be different from that of a drug acting at sites containing receptors responsive to both serotonin and LSD Or certain drugs, such as butabarbital and meprobamate, might act to sensitize serotonin receptors or otherwise change the conditions there, with the result that previously inactive LSD might be activated and produce a serotoninlike effect However, the results obtained here do not support such speculation, at least not in the present state of knowledge of the sites of action of these drugs On the contrary, it is noteworthy that differences in interaction with LSD and serotonin occur between drugs of the same general classifications

While the observations reported here are not readily explained in terms of current concepts of central serotonin function, they appear to reduce the plausibility of the idea that the psychological effects of LSD result from a simple antagonism between it and serotonin, such as seems to occur peripherally *in vitro*

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Prediction of Stability in Pharmaceutical Preparations[1] V*

The Solution Degradation of the Antibiotic Streptovaricin[2]

By EDWARD R. GARRETT

The prediction of stability and pharmaceutical formulation of compounds of unknown structure are especially important in the antibiotic age. The kinetics of solution degradation of the antibiotic complex of the structurally unknown streptovaricins are studied spectrophotometrically and equations obtained to characterize degradation as a function of pH. Bioassay has been correlated with spectra and conditions given for maximum stability of assay standards and preservation of yield. A mechanism is proposed that accounts for the available data and predicts the properties of degradation intermediates. The application of an analog computer to kinetic interpretation is given. Kinetic conditions for the differential assay of streptovaricin C were derived.

There is a need in pharmacy to study the changes in structures of molecules of unknown structure.

This apparently contradictory circumstance has become more evident with the advent of the antibiotic age. The application, efficacy, and toxicity of an antibiotic or antibiotic complex is frequently well known before the components can be separated, purified, individually characterized, and structures assigned. Pharmacy is often called upon to apply its unique talents in formulation and stabilization before the assignment of structure, perhaps even before ultimate purification.

The stability of materials must be known for the establishment of treatment of assay standards and for the preparation of samples to be submitted for clinical evaluation. Yet no know-how may be available as to what part of the molecule is hydrolyzed, pyrolyzed, or photolyzed; or how such degradations may be easily and quantitatively followed.

In general, the primary criteria of efficacy for compounds with such histories will be bioassays. However, bioassays as criteria of stability involve considerable expense, large error, and special isolation techniques that are frequently tedious and complex.

Prediction of stability and design of formulations may be based on studies of the rates of change of some physicochemical characteristic. Correlation of changes in the physicochemical properties with the changes in the bioassays may serve as a valid argument for the use of the former. An obvious disadvantage is that variation in the latter may not necessarily reflect in the former although the converse is not probable.

Obvious advantages are that such physicochemical studies will be easy to conduct, less expensive, can lead to prediction of the conditions that affect degradation and provide information on the properties of the intermediates and products. These facts can contribute to process design for maximum yield, the achievement of stable standards, and the least degrading assay conditions.

Examples of the application of such a philosophy were the stability studies on the antibiotic fumagillin (2, 3, 4).

The antibiotic complex of the streptovaricins of unknown structure is a good case in point. The complex is of particular interest because of its high antitubercular activity (5, 6, 7), yet it is a complex of closely related antibiotics with no apparently distinguishable ultraviolet spectra and only slightly variable infrared spectral differences (8). The streptovaricin complex consists of at least five components, A, B, C, D, and E (5, 8), separable by the tedious processes of paper chromatography (9) and countercurrent extraction (8).

This paper reports on studies designed to determine the total and comparative stabilities of the streptovaricin components as a function of pH, to predict the nature of the intermediates or products of degradation from the physicochemical changes, to provide clues for easier separation or easier assay by differential degradation, and to predict the conditions for maximum stability in solution.

EXPERIMENTAL

Four components of the streptovaricin complex, i.e., A, B, C, and D, were isolated by chromato-

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[1] See reference (1) for prior paper in this series.

[2] The trade name of The Upjohn Co. for the antimicrobial streptovaricin is Dalacin.

graphic column and subsequent Craig countercurrent distribution according to the procedure of Herr, *et al* (8) The compounds used were prepared by these authors

The general procedure for the ultraviolet spectrophotometric studies on the solution degradation of the streptovaricins was to weigh out the necessary amount of material (80 mg) and take up to volume in a 500 ml volumetric flask with absolute ethanol maintained in a 30 3° constant temperature bath For each buffer solution, 25 ml of the ethanolic solution was pipetted into a 100 ml volumetric flask and brought up to volume with the appropriate buffer, previously equilibrated at the constant temperature The final concentration was 40 μg/ml in the streptovaricin and the final buffer concentration is given in Table I

The resultant solutions were run at various intervals on the Cary recording ultraviolet spectrophotometer, Model 11, against a 25% ethanol buffer blank made up at the same time as the streptovaricin solutions

The pH values were checked immediately and at intervals on a Cambridge pH meter, equipped with glass saturated calomel electrodes The pH values were consistent throughout the runs and averages are given in Table I

Spectrophotometric readings were also made on the streptovaricin solutions in absolute ethanol and on nitrogen purged, buffered aqueous ethanolic solutions No significant spectral change was observed in the former case and nitrogen purging had no retarding effects on the degradation rates in the latter case

Material submitted for bioassay against *S lutea* (9 10 11) was diluted to the appropriate concentration range When the degradation was in the highly alkaline or acidic regions the samples taken for bioassay were immediately neutralized so as to stabilize the streptovaricins submitted

In order to study the effects of varying salt concentrations and check the possibilities of general acid-base catalysis the pH was maintained constant but the acetate acetic acid buffer concentration was varied (See Trials 11, 12, and 13 in Table I) No significant change in degradation rate was observed

RESULTS

Streptovaricin C Degradation as Characterized by the 432 and 316 mμ Chromophores—The ultraviolet spectra of streptovaricin are given by Curve A, Fig 1 The spectra are independent of pH within the range 1 0–9 0 Representative wavelengths characteristic of the spectrum are the 432 mμ maximum, 316 mμ plateau, the shoulder maximum at 263 mμ, and the major maximum at 244 5 mμ

Within the pH range 1 0–9 0, the absorptivity at the 432 mμ maximum decreases with time to an asymptotic value at apparently the same rate as does the absorptivity at the 316 mμ plateau The rate constants of the apparent first order degradation may be determined by application of the expression to the absorbance

$$\log(A - A_\infty) = -kt/2.303 + \log(A_0 - A_\infty) \quad \text{(Eq 1)}$$

where A_0 is 0 600 at 432 mμ and 0 615 at 316 mμ and

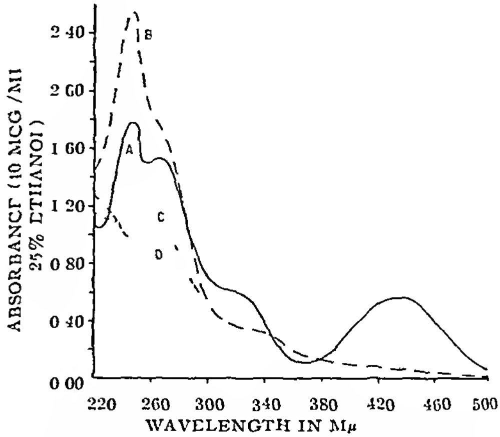

Fig 1—The ultraviolet spectra of streptovaricin C and degradation products Curve A—all streptovaricins, Curve B—first product of degradation in acid solution, i e, streptovaricin C′ in 0 1 *M* HCl, Curve C—final product of acid degradation and acidified neutral degraded material, i e, streptovaricin C″ in 0 1 *M* HCl solution, Curve D—final product of neutral degradation and neutralized acid degraded material, i e, streptovaricin C″ at pH 7 4

where A_∞ is 0 100 at 432 mμ and 0 320 at 316 mμ for 40 μg streptovaricin C per ml of 25% ethanol The given A_∞ at 316 mμ is only valid above pH 2 00 The absorbance, A, is at a time, t

Apparent first order plots in accordance with Eq 1 for absorbance changes at 432 mμ at representative pH values are given in Fig 2 The estimated rate constants (k in sec $^{-1}$) are given in Table I and a plot of log k *vs* observed or actual pH is given in Fig 3 Figure 3 clearly shows that the rate of degradation of streptovaricin C as characterized by the 432 mμ chromophore disappearance is hydrogen ion dependent below pH 3 and a good estimate of the rate (k in sec $^{-1}$) below pH 3 is

$$\log k = -\text{pH} - 3.400 \quad \text{(Eq 2)}$$

Thus, the apparent mechanism of degradation of streptovaricin C from 432 and 316 mμ observation is

$$C \xrightarrow{k_H{}^+ [H^+]} C' \quad \text{(Eq 3)}$$

where the absorptivities of C are $a_{432} = 15{,}000$, $a_{316} = 15{,}600$, and of C' are $a'_{432} = 2{,}500$, $a'_{316} = 8{,}000$

If the data on varying HCl molarity is considered (trials 1–7, Table I), then

$$k = 3.27 \cdot 10^{-4}[H^+] + 11.8 \cdot 10^{-7} \quad \text{(Eq 4)}$$

This implies that a "water" or spontaneous reaction rate constant, k_0, is 11 8 10^{-7} sec $^{-1}$ which is characterized by the horizontal solid line in Fig 3 The increased rate above this solid line above pH 7 could be attributed to some hydroxyl ion attack, the lower rate at pH 3 25 is difficult to interpret and may or may not be real The asymptotic value at 316 mμ appears to be a minor function of the pH of degradation and/or the pH of the media (see Curves C and D in Fig 1) Since a mean A_∞ was assumed constant for this wavelength, less emphasis should be placed

TABLE I—APPARENT FIRST ORDER RATE CONSTANTS (k IN SEC $^{-1}$) FOR THE LOSS OF THE 432 AND 316 mμ CHROMOPHORES OF STREPTOVARICIN C[a]

Trial	pH	Buffer Composition	10 k (sec $^{-1}$) 432 mμ	10 k (sec $^{-1}$) 316 mμ
1	1 19	0 075 M HCl	243	
2	1 30	0 060 M HCl	207	
3	1 47	0 040 M HCl	144	
4	1 77	0 020 M HCl	77 8	
5	2 04	0 010 M HCl	44 2	
6	1 12	0 0375 M KCl, 0 0783 M HCl	258	119
7	1 97	0 375 M KCl, 0 0125 M HCl	48 2	30 7
8	2 73	0 427 M CH_3COOH	9 80	7 57
9	3 24	0 0427 M CH_3COOH	5 60	4 65
10	4 38	0 1000 M CH_3COOH, 0 025 M CH_3COONa	10 5	10 8
11	4 99	0 0264 M CH_3COOH, 0 02425 M CH_3COONa	12 8	14 3
12	4 99	0 0532 M CH_3COOH, 0 0500 M CH_3COONa	13 5	15 7
13	4 98	0 1063 M CH_3COOH, 0 1000 M CH_3COONa	12 8	14 7
14	5 33	0 123 M CH_3COOH, 0 4000 M CH_3COONa	12 3	13 2
15	5 41	0 0244 M CH_3COOH, 0 0628 M CH_3COONa	13 1	14 4
16	5 88	0 0210 M CH_3COOH, 0 2000 M CH_3COONa	8–12	10 0
17	6 22	0 0030 M CH_3COOH, 0 0628 M CH_3COONa	10–12	9 96
18	7 42	0 0948 M KH_2PO_4, 0 0568 M NaOH	15 1	9 19
19	8 57	0 0777 M KH_2PO_4, 0 0745 M NaOH	15–19	11 6
20	7 08	43 8% ethanol, no buffer	4 21	4 51

[a] At 40 μg /ml in 25% ethanol at 30 3°

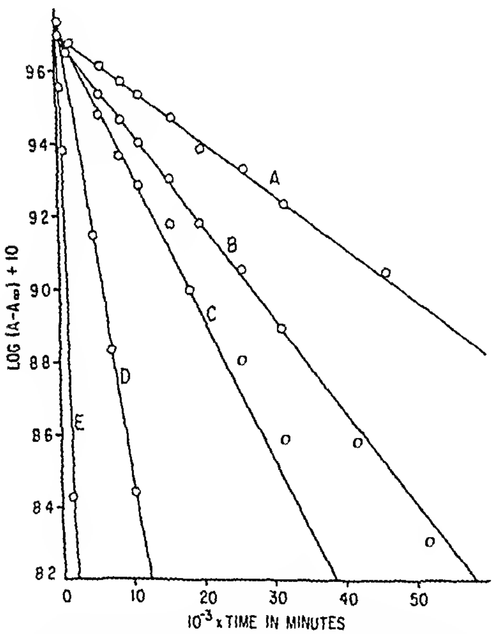

Fig 2—Apparent first order disappearance of the 432 mμ absorbance for streptovaricin C at 40 μg /ml of 25% ethanol at 30 3° as studied at varying pH values Curve A—pH 3 24, Curve B—pH 2 73; Curve C.—pH 7.42, Curve D—pH 1 97, Curve E.—pH 1 12.

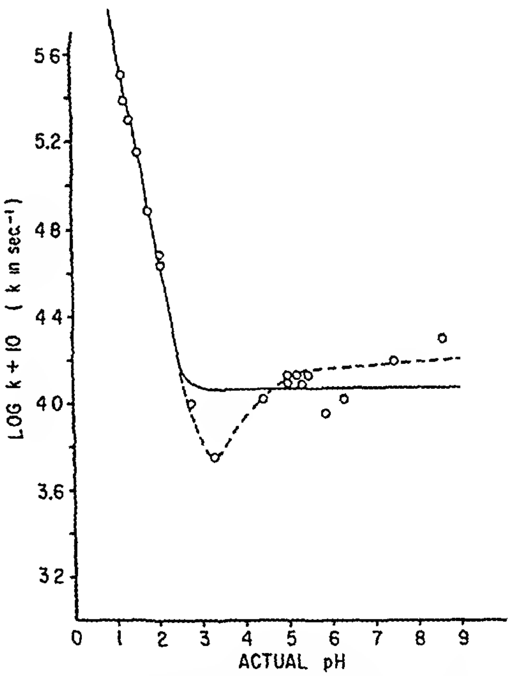

Fig 3—The pH profile of the degradation rates of streptovaricin C based on the 432 mμ absorbance disappearance in 25% ethanol at 30 3°.

on the reliability of the rate constants calculated at 316 mμ Comparison of rates of degradation of nitrogen purged and nonpurged solutions showed no differences This indicates that air oxidative processes under these conditions were not responsible

Streptovaricin C Degradation Characterized by Lower Wavelength Chromophores.—Streptovaricin C at low pH values shows an increase and then a subsequent decrease in absorbance at the low wavelengths, i e , at 245 mμ and 263 mμ The rate of increase at 245 mμ corresponds to the rate of decrease at 432 mμ Figure 4 graphically shows this to be so in that the apparent maximum at 235 mμ correlates with the maximum loss in absorptivity at 432 mμ for pH values below 3.

The spectra of acid degraded streptovaricin C at approximately the time that the maximum absorbance was reached at 245 mμ is shown in Fig. 1, Curve B, and in Fig 5, Curve A. The latter was obtained after twenty hours at 30° in 0.075 M HCl in 25% ethanol at 40 μg /ml The absorbance is at 12 6 μg /ml in 25% ethanol at pH 0.8 This may be an isolable intermediate, C', where the increased ab-

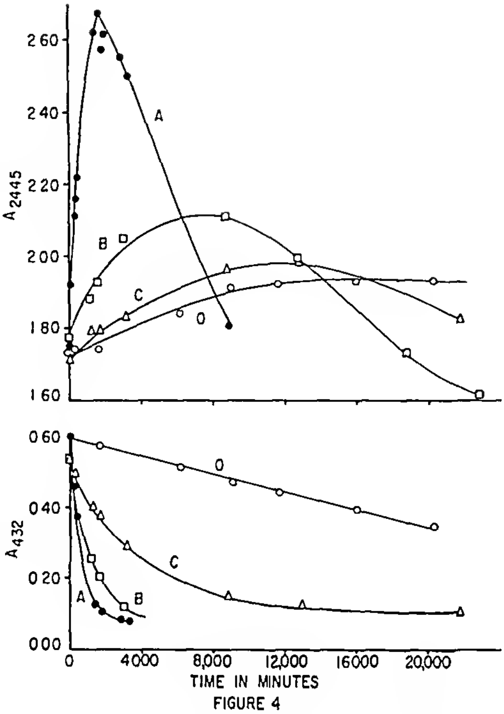

Fig 4 —Demonstration of the correspondence in time of the absorbance, A_{245}, increase to a maximum at 245 mμ and the absorbance, A_{432}, decrease to a minimum at 432 mμ of streptovaricin C at 40 μg /ml 25% ethanol at 30 3 ° Curve A —pH 1 12 Curve B —pH 1 47, Curve C —pH 2 04, Curve D —pH 3 24

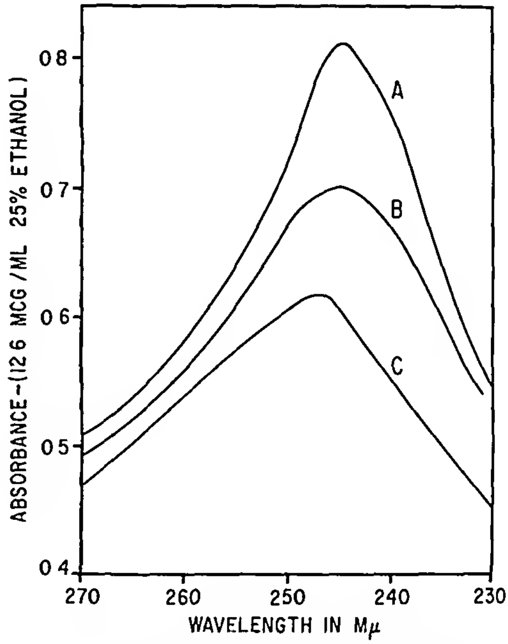

Fig 5 —Base acid reversible spectra of streptovaricin C′, an intermediate in streptovaricin C acid degradation Curve A —streptovaricin C′ and reacidified neutralized and buffered C′, Curve B —C′ from acid degradation adjusted to pH 4 33 Curve C —C′ from acid degradation adjusted to pH values of 5 4, 6 1, 7 4, and 8 2

sorbance at 245 mμ washes out the still present subsidiary maximum at 263 mμ The latter absorption in Fig 1, Curve B, does not appear to be significantly altered in intrinsic intensity by the transformation

The intermediate, C′, exhibited a decrease in absorbance of the 244 5 mμ when adjusted to pH values in excess of 2 5 Curve A, Fig 5, is not only the spectra of C′ but of C′ obtained from neutral degradations adjusted to pH values of 1 2 and 2 4 Curve B, Fig 5, is the spectra of C′ adjusted to pH 4 33 whereas Curve C is adjusted to pH's of 5 40, 6 05, 7 39 and 8 24 by sodium hydroxide and acetate buffers Curve A is also the result when these latter solutions are acidified with HCl to obtain the original acid conditions

These facts permit the conclusion that the acid degradation to C′ has produced a structure with chromophoric enhancement at 245 mμ and chromophoric loss at 432 mμ The base acid reversibility of the C′ chromophore changes at 245 mμ can be attributed to a C′ functional group of pKa *ca* 4

The intermediate, C′, however, exhibited a decrease in the 245 mμ absorbance and increases in 263 mμ and 360 mμ absorbances, as well as a visible color when adjusted to pH values in excess of 10 The color cleared on reacidification, but the 245 mμ absorbance was slightly higher than its original value This indicates a pH susceptible group in C′ of pKa *ca* 10 which, in part, is irreversibly affected

The subsequent decrease in absorbance at 245 mμ after the maximum absorbance (corresponding to C′) at low pH values tends to give a final product (Curve C in Fig 1) with a slight maximum at 263 mμ This absorbance decrease is apparently subsequent to the elimination of the 432 mμ chromophore and, as will be shown later, is subsequent to the destruction of the bioactivity Figure 6 shows the absorbance at 245 mμ against time as a function of pH of degradation The absorbance of the maxima is less when the reaction is studied at higher pH values Above pH 4 no maximum is observable in such plots

The spectra of completely degraded C does approach asymptotic values In the neutral pH region the asymptotic spectra has a mild maximum at 237 mμ (See Curve D in Fig 1) When acidified, this mild maximum is lost but one is gained at 263 mμ which very closely approximates the asymptotic spectra of acid degraded C (See Curve C in Fig 1) Conversely, acid degraded materials (Curve C) when neutralized have spectra closely approximating the asymptotic spectra of neutral degraded material (Curve D in Fig 1) They are not exactly superimposable but the major chromophores are retained

Chromophoric Degradation of Streptovaricins A, B, C, and D.—The ultraviolet spectra of undegraded streptovaricin C is independent of pH and the spectra of all streptovaricins are similar except for a small displacement in absorptivity which could be attributed to slight impurities

The rates of degradation of streptovaricins A B, C, and D were compared at pH 1 1 and 8 5 as measured at 245, 260, 316, and 432 mμ, all at 30 3° at 40 μg /ml and in 25% ethanol

At pH 1 1, the previously discussed initial in

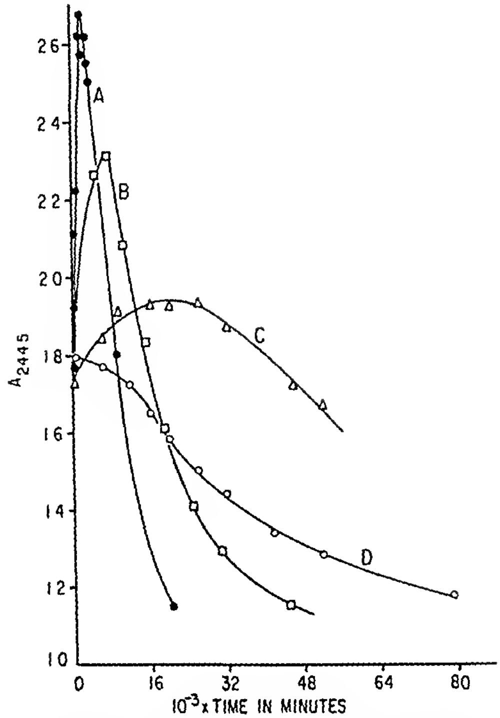

Fig. 6.—Plot of the absorbance at 244.5 mμ of streptovaricin C with time at various pH values. Curve A—pH 1.12; Curve B.—pH 1.97; Curve C.—pH 3.24; Curve D.—pH 4.38.

crease and subsequent decrease in absorbance at 244.5 mμ and 260 mμ were noted. Except for the expected vertical displacement, the plots of $A_{244.5}$ *vs.* time for the various streptovaricins were parallel (See Fig. 7). The first order plots of 432 and 316 mμ absorbance disappearance showed no gross difference in rate (See Fig. 8) except for a slightly faster degradation rate for streptovaricin D.

At pH 8.5, the same conclusions of similar chromophoric degradation could be made.

Correlation of Bioactivity and Chromophore Degradations.—The concentration of degrading streptovaricin, $[S]$, in 25% ethanol may be computed from the absorbance at any time (A_t) at 432 mμ, the initial absorbance (A_0) of the initial concentration of streptovaricin, $[S]_0$, and the asymptotic absorbance (A_∞) of the degradation product S' where $[S']_\infty = [S]_0$:

$$[S] = \frac{A_t - a'[S]_0}{a - a'} \qquad \text{(Eq 5)}$$

where $a = A_0/[S]_0$ is the absorptivity of the streptovaricin and $a' = A_\infty/[S]_0$ is the absorptivity of the final product $[S']_\infty$. For streptovaricin C, $a = 15{,}000$; $a' = 2{,}500$ at 432 mμ.

Streptovaricins in Acid.—The streptovaricin C concentration degraded in 0.075 *M* HCl and as calculated from Eq. 5 at 432 mμ is plotted against the bioassay results in Fig. 9. The correlation, in gen-

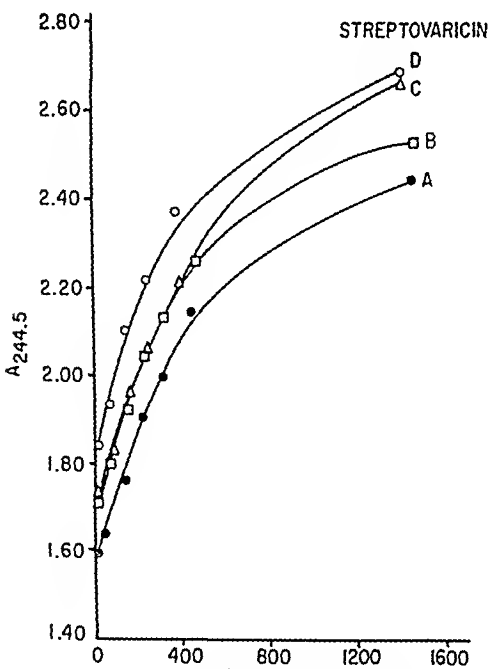

Fig. 7.—Spectral absorbance increase to a maximum with time at 244.5 mμ for various streptovaricins at pH 1.2 in 25% ethanol with HCl-KCl buffer.

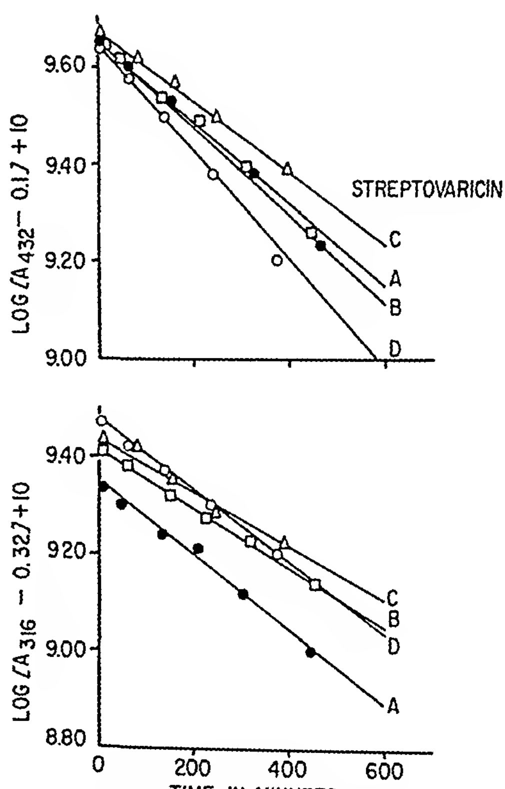

Fig. 8.—Pseudo first order plots of spectral absorbance changes at 316 mμ and 432 mμ for various streptovaricins at pH 1.2 in 25% ethanol with HCl-KCl buffer.

eral, is good The aliquots submitted for bioassay were adjusted to pH 3–4 after sampling since degradation rate appeared to be lowest in this pH range

Similar correlations were possible for streptovaricins A and B when these were subjected to acid degradation, i e, at pH 1 0 However, streptovaricin D demonstrated a faster decrease in 432 mμ chromophore than in apparent activity

Streptovaricins at Neutral pH —Streptovaricin C degradation at pH 5 00 (Fig 10) and similarly at pH 8 5 showed good correlation of bioactivity with concentration calculated from the 432 mμ chromophore At pH 8 5, however, the other streptovaricins acted anomalously and showed gross deterioration in bioactivity for little change in spectra, completely losing their potency by bioassay within a maximum of eight hours, whereas streptovaricin C only lost 6% as calculated from the rate constants of Table I

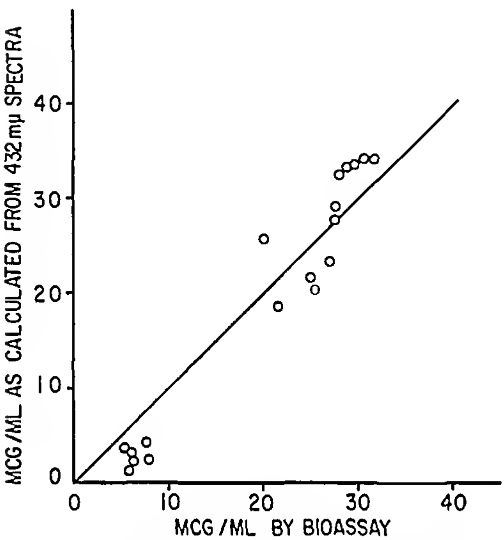

Fig 9 —Correlation of streptovaricin C concentration in 25% ethanol calculated from 432 mμ absorbance and concentration based on bioassay The samples were taken at various times in 0 075 M HCl and adjusted to pH 4 on sampling The line represents the theoretical relation

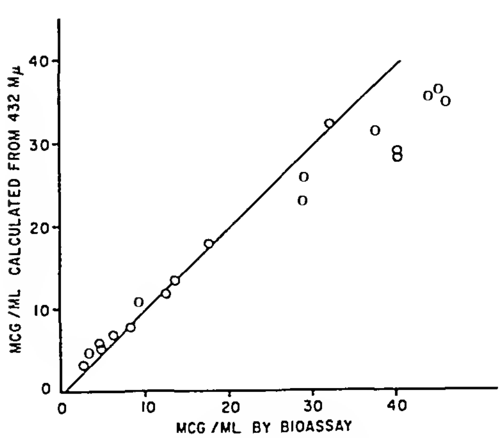

FIGURE 10

Fig 10 —Correlation of streptovaricin C concentration in 25% ethanol calculated from 432 mμ absorbance and concentration based on bioassay The samples were taken at various times in pH 5 0 acetate acetic acid buffer The solid line represents the theoretical relation

Streptovaricins in Alkali.—Degradation of streptovaricin C in 0 01 M to 0 06 M NaOH results in a definite portion of the initial absorbance being destroyed almost instantaneously, *ca* 40% at 244 5 and 263 mμ, and *ca* 20% at 316 and 432 mμ Thereafter, the change in absorbance is extremely slow

The studies of change in bioassay of streptovaricins A, B, C, and D in 0 01 M NaOH were conducted by neutralizing an aliquot with 0 01 M HCl and buffering at pH 3 The biological activity was completely gone within fifteen minutes The estimated half life of this activity was five minutes at this alkalinity

DISCUSSION

Possible Mechanism to Explain Streptovaricin C Degradation —A postulated reaction sequence with equilibria that fit the results from the spectrophotometric studies of the acid and neutral degradation of streptovaricin C are

$$C \xrightarrow{k\ =\ k_{H^+}[H^+]\ +\ k_o} \left\{ +H^+ \begin{matrix} C'_1 \\ \uparrow\downarrow \\ C'_2 \end{matrix} -H^+ \right\} \xrightarrow{k'} C'' \qquad \text{(Eq 6)}$$

where the evaluated constants for k are given in Eq 4 and $C' = C'_1 + C'_2$

This scheme was substantiated by the following kinetic treatment of the experimental data

A series of degradation studies of 40 μg streptovaricin C per ml of 25% ethanol were conducted by following the absorbance changes with time at 245 mμ These data which included the examples given in Fig 6 were analyzed by determining the first order rate, k', of approach to an asymptotic absorbance after the appearance of the maximum absorbance at this wavelength, i e, for the postulated $C' \rightarrow C'$ transformation A value for 1 0 for A_∞ was reasonably constant for all studies and the k' in sec $^{-1}$ were determined from plots of log $(A - A_\infty)$ *vs* time as per Eq 1 The k' values so determined are listed in Table II as well as the k values determined from the complete loss of the 432 mμ chromophore in the same study The rate constant k in sec $^{-1}$ for the postulated $C \rightarrow C'$ transformation is also considered as the rate constant characterizing the appearance of the 245 mμ maximum observed at the lower pH values since the parallelism of the two spectral changes with time was so apparent

The total absorbance, A_t at 245 mμ at any time is made up of the contributions from the nondegraded streptovaricin C, the intermediate C' either in the protonated form, C'_1, or the nonprotonated form C'_2 and the final product C'' as based on Eq 6 Also, where $(C)_o$ is the initial concentration of streptovaricin C

$$[C]_o = [C] + [C'] + [C''] \qquad \text{(Eq 7a)}$$

$$[C'] = [C_1'] + [C_2'] \qquad \text{(Eq 7b)}$$

The respective absorptivities are a, a', a'_1, a'_2, and a'' so that

$$A_t = A + A' + A'' \quad \text{(Eq. 8a)}$$

$$A_t = a[C] + a'[C'] = (a_1'[C_1'] + a_2[C_2']) + a''[C''] \quad \text{(Eq. 8b)}$$

$$A' = a_1'[C_1'] + a_2'[C_2'] \quad \text{(Eq. 9)}$$

If the equilibrium between C'_1 and C'_2 is hydrogen ion dependent, then

$$[C_2'] = [C_1']K'/[H^+] \quad \text{(Eq. 10)}$$

Thus, substitution of Eq. 10 into Eqs. 7b, and 9 and division of Eq. 9 by 7b result in an equation for the apparent absorptivity, a', of C':

$$a' = A'/[C'] = \frac{a_1' + a_2'K'/[H^+]}{1 + K'/[H^+]} \quad \text{(Eq. 11)}$$

It follows that, at high pH or low $[H^+]$,

$$a' \rightarrow a_1'[H^+]/K' + a_2' \rightarrow a_2' \quad \text{(Eq. 12)}$$

and that, at low pH or high $[H^+]$,

$$a' \rightarrow a_1' + a_2K'/[H^+] \rightarrow a_1' \quad \text{(Eq. 13)}$$

so that the apparent absorptivity, a', varies between a_1' and a_2' and at equivalent amounts of C_1' and C_2' where, in general, $[H^+] = K'$ from Eq. 10, then

$$a' = (a_1' + a_2')/2;\ [H^+] = K' \quad \text{(Eq. 14)}$$

The absorptivities of C and C'' can be determined from the initial and final spectral absorbances at 245 mμ, i. e., $a_{245} = A_0/[C]_0 = 46{,}000$ and $a''_{245} = A_\infty/[C]_0 = 26{,}000$. The k and k' values for a given pH are also known since the former is the pseudo first order rate constant for the disappearance of the 432 mμ maximum and equivalent to the rate of appearance of the 245 mμ maximum absorbance which appears as an intermediate in that chromophore's change with time.

The curves of 245 mμ absorbance against time as given in Fig. 6 can be fitted by an electrical analog wherein the model is based on Eqs. 6–9. The potentiometer settings to give the best fit can be translated into k, k', a, a', and a'' values. The results of this curve fitting by the use of an analog computer are given in Table II.

Since the manipulation of one potentiometer setting necessitated changes in all the others, this fitting was not an easy procedure. Thus, the striking correlation in Table I of the analogously computed estimates of k (the rate constant for the appearance of C' as a maximum absorbance at 245 mμ) and those determined graphically from the pseudo first order decrease of the absorbance at 432 mμ (see Fig. 2) is considered to be highly significant. The analogously computed estimates of k' (rate of appearance of C'' as an asymptotic value at 245 mμ) and those determined graphically from the pseudo first order decrease of the 245 mμ maximum are also highly comparable.

It is concluded that the mechanism proposed in Eq. 6 is consistent with the experimental data. The analogously computed estimates of the absorptivities a, a', and a'' are also given in Table II. The a and a'' values are constant with change in pH, indicative of the absence of any functional groups affected by pH, and interacting with the chromophores. The a' values are not constant, indicative of the converse situation. They range from a value of 80,000 at pH 1.0 to 44,000 at pH values in excess of 6. Thus the a'_1 absorptivity may be assigned to the former numerical value and a_2' to the latter. The pKa of the C' functional group may be estimated by Eq. 14 as *ca.* 3.5. This value is consistent with the pKa previously estimated from the reversible 245 mμ absorbance changes of the solution of the C' intermediate.

Applications of Results of Kinetic Studies.—The results of these studies have great pharmaceutical utility. Below a pH of 7, the absorbance at the 432 mμ chromophore can be used as a valid estimate for the biological potency of streptovaricin, $[S]$, by the expression

$$[S] = \frac{A - a'[S]_o}{A - a'} = \frac{A - 2{,}500[S]_o}{12{,}500} \quad \text{(Eq. 15)}$$

where $[S]_o$ is the initial concentration in grams/liter and A is the absorbance at 432 mμ. Since the absorbance of the 432 mμ chromophore of streptovaricin C can be so correlated with the loss in biological activity below a pH of 9, the kinetic studies of the former can be used to calculate streptovaricin C stability at any pH ($-\log [H^+]$) below 9 at 30.3°. The appropriate equation is

$$\log \text{(fraction of potency remaining)} = -kt/2.303 \quad \text{(Eq. 16)}$$

where k in 25% ethanol is given by Eq. 4.

Estimates of retention of potency in the stomach are possible. If pH values are assumed for the stomach, the half-life of streptovaricin C in the stomach is less than six hours for pH 1.0 and less than forty-

TABLE II.—APPARENT FIRST ORDER RATE CONSTANTS (IN SEC.$^{-1}$) AND ESTIMATES OF ABSORPTIVITY AT 244.5 mμ FOR THE DEGRADATION OF STREPTOVARICIN $C \xrightarrow{k} C' \xrightarrow{k'} C''$

pH	Analog Computer Estimates of Absorptivity $10^{-3}\,a^a$	$10^{-3}\,a'^b$	$10^{-3}\,a''^c$	Analog Computer Estimates 10^7k	$10^7k'$	First Order Plots 10^7k^d	$10^7k'^e$
1.12	46	80	25	160	25	260	18
1.97	46	74	26.5	53	12.5	48	9.8
2.73	47	62	27	16	3.7	9.8	3.2
4.38	46	48	26	27	..	10.5	3.9
5.41	45	45	26	17	..	13	5.5
6.22	46	44	26	17	..	12	5.7
7.42	46	39	29	24	..	15	7.4
8.57	46	44	26	17	.	17	6.5

[a] Absorptivity of C. [b] Absorptivity of C'. [c] Absorptivity of C''. [d] Based on first-order plots of absorbance disappearance at 432 mμ. [e] Based on first-order approach to final asymptote at 244.5 mμ.

three hours for pH 2. The effect of alcohol content, in general, is to decrease hydrolysis rates as is shown in the shift of k in sec $^{-1}$ from 12×10^{-7} to 4.5×10^{-7} for an increase in alcohol content from 25% to 44% (see Table I). It may be concluded that significant loss of streptovaricin potency will occur in gastric contents below pH 1.5 if the stomach emptying time is assumed to be four hours.

It is obviously mandatory to refrigerate or freeze standard solutions and to extract or assay solutions in minimum time since the half-life of streptovaricin C should not be considered as more than fifty-three hours as calculated at 30° in 25% ethanol. However, standard solutions of high stability could be made in absolute alcohol.

A maximum yield of a hydrolytic intermediate, C', characterized by the 245 mμ maximum absorbance could be obtained in twenty-four hours in 0.1 M HCl, 25% ethanol at 30°.

An assay for streptovaricin C could be developed by permitting the streptovaricin complex of A, B, C, and D of concentration $[S]$ to stand in 25% ethanol at pH 8.5 for eight hours at 30°. The final bioassay should be largely streptovaricin C of concentration $[C]$. The original streptovaricin C concentration, $[C]_0$, of the solution could be calculated from

$$\log [C]_0 = \log [C] + kt/2.303 \quad \text{(Eq. 17)}$$

where t is in seconds and k is 15×10^{-7} sec $^{-1}$. The fraction, f, of C in the original complex could be estimated from

$$f = [C]_0/[S] \quad \text{(Eq. 18)}$$

This unique difference in the maintenance of bioactivity in aqueous solution provides an excellent rationale for the fact that C is the observed major component in the streptovaricin complex (8).

Thus, although the streptovaricins retain their structural anonymity, the observations on their physicochemical transformations permit the prediction of their routes and how to restrict their rates of travel.

SUMMARY

1. Streptovaricin C is degraded by specific hydrogen ion catalyzed and "spontaneous" hydrolysis. The degradation was followed by the loss of absorbance at 432 mμ and the appearance of a maximum absorbance at 245 mμ. The former in solutions below pH 9 is correlated with the loss in biological activity and can be used to calculate the nonhydrolyzed antibiotic remaining. Equations and constants are provided to calculate stability under various pH conditions.

2. General acid-base catalysis and oxidative degradation in solution were not observed. Increased alcohol content stabilized the streptovaricins; high stability results in absolute ethanol.

3. Streptovaricin C degrades to a biologically inactive intermediate C' characterized by enhanced absorbance at 245 mμ in acid. This absorbance is reversibly lost on neutralization indicative of a pKa *ca.* 3.5–4.0. An additional pKa *ca.* 10 was also indicated. This C' is further hydrolytically degraded with a loss of absorbance at all wavelengths to C'' and rate constants for this transformation have also been determined. A maximum yield of C' should be obtained in twenty-four hours in 0.1 M HCl and 25 per cent ethanol at 30°.

4. An assay for streptovaricin C could be developed by permitting the streptovaricin complex of A, B, C, and D to stand in 25 per cent ethanol for eight hours at pH 8.5 and at 30°. The final bioassay should be largely C. The original C concentration could be calculated from the data given.

5. In 0.01 N NaOH at 30°, the half-life of the streptovaricins is less than five minutes. The mechanism of alkaline degradation appears to differ from that of acid and "spontaneous" hydrolyses.

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The Solubility of Various Sulfonamides Employed in Urinary Tract Infections*

By F. J. BANDELIN† and WALTER MALESH

The solubilities of sulfonamides commonly employed in the therapy of urinary tract infections and their N⁴ acetyl derivatives have been investigated in phosphate buffers and in a synthetic urine known as Mosher's Vehicle over the pH range 4.5 to 7.0 at 37°. All compounds increased in solubility with increasing pH and all had similar curve characteristics. Of the compounds investigated, sulfacetamide exhibited the greatest solubility, followed in decreasing order by sulfaethylthiadiazole, sulfamethylthiadiazole, sulfisoxazole, and the trisulfapyrimidines. The solubility characteristics of sulfamethoxypyridazine were found to differ from the other compounds of this group. The N⁴ acetyl derivatives exhibited solubility curves similar to those of the parent compound but were generally less soluble in the media employed, with the exception of the combined trisulfapyrimidine acetyl derivatives. As a general rule, both the sulfonamides and their acetyl derivatives were more soluble in Mosher's Vehicle than in the phosphate buffers. Comparison of solubility data is made and details of the experimental methods given.

THE INSOLUBILITY of early sulfonamides and their acetyl derivatives in water and in tissue fluids, and the hazards of sulfonamide urolithiasis caused by the lack of solubility of these compounds had led to the development and introduction of sulfonamide compounds and mixtures of greater solubility and less danger of crystalluria.

Lehr (1) in 1945 introduced the mixed sulfonamides and with subsequent work (2–6) illustrated the value of the principle of separate solubilities for increasing the solubility of sulfonamides by such mixtures. This work was corroborated by Frisk and associates (7, 8) and by Oettingen and Cronheim (9) and led to the acceptance, in 1949, by the Council on Pharmacy and Chemistry of the American Medical Association of preparations containing two (10) or three (11) sulfonamides.

Soluble sulfonamides such as sulfacetamide, sulfamethylthiadiazole, and sulfisoxazole have become widely used as single entities and in combinations.

The essentiality of solubility for sulfonamides recommended for urinary infection has been stressed by a number of clinicians. Seneca, Henderson, and Harvey state (12), "In making a choice of sulfonamides for the treatment of urinary tract infections, a highly active, absorbable, and soluble compound which is excreted in high concentration in the urine is obviously sought." O'Connor (13) also states, "Sulfonamides of improved urinary solubility offer the cheapest and most easily tolerated drugs for routine office prescription."

Hawking and Lawrence state (14), "In determining the relative value of the sulfonamide compounds in urinary infections, numerous factors are concerned. The effectiveness depends upon the relative potency of the compound, on its concentration in the urine determined by the rate of excretion, solubility, on the degree of acetylation in the urine, and on the concentration in the kidney, ureter, and bladder wall."

Marshall (15), Yow (16), Tice (17), and Bourque and Joyal (18), among others, agree on the desirability of compounds of high solubility.

That high blood levels are not necessary to obtain a bactericidal urinary concentration with sulfonamides has been pointed out by Herrold (19), Cook (20), and others.

Since sulfonamides generally have a greater antibacterial effect against Gram-positive cocci than against Gram-negative bacilli such as *E. coli*, *Proteus*, *A. aeruginosa*, and *Ps. aeruginosa*, and since it is these latter which are largely responsible for urinary tract infections, it follows that higher concentrations of sulfonamide are required to inhibit these organisms.

In consideration of these various factors we investigated and determined the solubilities of sulfonamides generally recommended for urinary tract infections and their acetyl derivatives, both in phosphate buffer and in a synthetic urine, over the usual pH range of the urine (pH 4.5 to 7.0) and have plotted curves and derived values for comparison of solubilities at increasing pH increments as hereinafter described.

EXPERIMENTAL

Preparation of Sulfonamide and Acetyl Sulfonamide Solutions in Buffers.—The buffer systems employed in this work were essentially the phosphate buffers of Gortner (21) which were prepared at four times the concentrations given by the

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author.[1] The two solutions were mixed to obtain buffer solutions of precise pH values, increasing in 0.5 pH increments from 4.5 to 8.0 as required. The pH values of all buffer mixtures were determined with a pH electrometer and adjustments made, where necessary, to produce buffers of accurate values.

Solutions were prepared by adding an excess of the sulfonamide or its acetyl derivative to 10 ml of buffer solution at each pH level in 18 x 150-mm. test tubes, stoppering the tubes, placing in a water bath at 37° with gentle agitation for twenty-four hours to allow equilibrium to be reached. At the end of this time, the mixture was filtered through a dry E and D No. 192 fluted filter paper, and a 1-ml. aliquot was accurately pipetted into a volumetric flask for dilution and analysis. The balance was retained for pH determination to ascertain any change in pH value This latter value was designated as the equilibrium pH. All solutions were prepared in duplicate, and duplicate samples were used for analysis throughout.

Preparation of Sulfonamide and Acetyl Sulfonamide Solutions in Synthetic Urine.—The synthetic urine employed, known as the Mosher Vehicle (22), is made up of the following per liter of water: sodium chloride 2.950 Gm., potassium chloride 1.660 Gm., magnesium chloride 0.121 Gm , calcium chloride 0.143 Gm., ammonium phosphate 0.300 Gm., and urea 20 Gm. To 10-ml. aliquots of this solution in 18 x 150-mm. test tubes at 37°, an excess of the sulfonamide or its aetyl derivative was added followed by sufficient one per cent H_3PO_4 or one per cent NaOH to adjust to the required pH value, thus producing solutions increasing by 0.5 pH increment from 4.5 to 7.0. The solutions were agitated in a 37° water bath for twenty-four hours. Meanwhile acid or base was added to keep the solutions at the desired pH level until equilibrium in pH and in concentration was attained. Since the Mosher Vehicle has little buffer capacity it was found necessary to adjust these solutions to the exact pH points of investigation otherwise the shift in pH due to the solubility of some of the sulfonamides was so great as to throw the final pH value outside of the range of investigation. After equilibrium for twenty-four hours at 37°, the solution was filtered as above and an aliquot pipetted into a volumetric flask for analysis.[2]

Sulfonamide Determination.—The colorimetric method employed for the determination of the sulfonamide content of the solutions was essentially that of Bratton and Marshall as described in detail by Biamonte and Schneller (23). Standard curves were prepared for the individual sulfonamides using accurately prepared standard solutions.

All solutions of the acetyl derivatives were hydrolyzed by refluxing with 5% sulfuric acid for one hour to liberate the free amino compound before determination.

Determinations were made by reading the absorbance of the solution, after color development, at 545 mμ with a spectrophotometer using a reagent blank. Values obtained were read from the standard curves with appropriate factoring for the dilution. All solubilities are stated in mg. per 100 ml. of solution.

All determinations were run in duplicate and the average values at resultant pH values are given in Table I. Values for the acetyl derivatives are plotted as free sulfonamides.

Comparative solubilities of sulfonamides at 0.5 pH unit increments over the pH range 4.5 to 7.0 in phosphate buffers at 37° as obtained by extrapolation of the solubility curves derived from data are given in Table II. The same information on the acetyl derivatives is given in Table III. Figure 1 is included to illustrate graphically the comparative solubilities given in Table II.

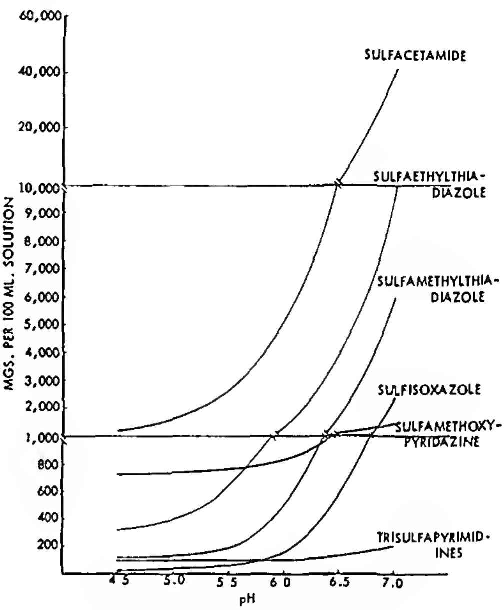

Fig. 1.—Solubilities of various sulfonamides in phosphate buffers at 37°.

DISCUSSION

As is apparent from the data presented, the differences in the solubilities of these various sulfonamides in the media employed, is considerable. The acetyl derivatives show similar differences in relative solubility, being less soluble than the parent compound with the exception of the trisulfapyrimidines, in which case the reverse is true as has already been demonstrated (23). As a general rule, both the sulfonamides and their acetyl derivatives were slightly more soluble in Mosher's Vehicle than in the phosphate buffers. This appears to be due to a direct solubilizing action of the urea upon the sulfonamide compounds.

While the sulfonamides have generally been considered as poorly soluble compounds, several of these compounds show marked solubility, especially at the higher pH values. Sulfacetamide, for instance, is soluble in excess of 40% at pH 7.0, while at this pH the remainder of the compounds had the following solubilities: sulfamethylthiadiazole 5.96%, sulfi-

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[1] Buffers consisted of varying mixtures of the following solutions: dibasic sodium phosphate, heptahydrate—71.6 Gm. per L. of distilled water; monobasic potassium phosphate—36.3 Gm. per L. of distilled water.

[2] At pH 7.0 some precipitation of calcium and magnesium hydroxides occurred in the synthetic urine. This was filtered off with the excess sulfonamide.

TABLE I —SOLUBILITY OF VARIOUS SULFONAMIDES AND THEIR ACETYL DERIVATIVES IN PHOSPHATE BUFFERS AND IN SYNTHETIC URINE OVER THE pH RANGE 4 5 TO 7 0

Milligrams of Compound Soluble in 100 ml of Buffer at 37°				Milligrams of Compound Soluble in 100 ml of Synthetic Urine at 37°			
Initial pH	Triple Sulfonamide	Equilibrium pH	Acetyl Triple Sulfonamide	Equilibrium pH	Triple Sulfonamide	Acetyl Triple Sulfonamide	Equilibrium pH
4 5	96	4 5	116	4 5	100	218	4 5
5 0	98	5 0	121	5 0	108	223	5 0
5 5	102	5 5	132	5 5	118	231	5 5
6 0	109	6 0	158	6 0	136	254	6 0
6 5	130	6 4	216	6 4	182	163	6 5
7 5	209	7 1	490	7 1	275	630	7 0
	Sulfacetamide		Acetyl Sulfacetamide		Sulfacetamide	Acetyl Sulfacetamide	
4 5			60	4 5	1,500	240	4 5
5 0	1,250	4 5	125	5 0	1,950	310	5 0
5 5	1,350	4 8	250	5 5	3,150	505	5 5
6 0	2,150	5 3	550	5 8	6,000	1,050	6 0
6 5	3,020	5 6	1,150	6 2	15,000	2,520	6 5
7 0	4,400	5 9	2,310	6 6	50,000	5,600	7 0
7 5	15,000	6 6	3,900	7 0			
8 0	41,000	7 0	3,900	7 0			
	Sulfamethyl-thiadiazole		Acetyl Sulfamethyl thiadiazole		Sulfamethyl-thiadiazole	Acetyl Sulfamethyl thiadiazole	
4 5	105	4 5	41	4 5	120	10	4 5
5 0	125	5 0	50	5 0	150	21	5 0
5 5	200	5 5	71	5 5	260	45	5 5
6 0	470	6 0	102	6 0	620	145	6 0
6 5	1,000	6 3	260	6 5	1,980	380	6 5
7 0	1,990	6 6	630	6 9	8,400	995	7 0
8 0	9,250	7 3	2,400	7 3			
	Sulfisoxazole		Acetyl Sulfisoxazole		Sulfisoxazole	Acetyl Sulfisoxazole	
4 5	33	4 5	8	4 5	36	30	4 5
5 0	45	5 0	12	5 0	51	44	5 0
5 5	70	5 5	38	5 5	80	70	5 5
6 0	175	6 0	105	6 0	220	160	6 0
6 5	405	6 4	190	6 4	710	560	6 5
7 0	1,360	6 8	375	6 7	2,600	1,230	7 0
7 5	2,870	7 2	1,040	7 1			
	Sulfaethyl-thiadiazole		Acetyl Sulfaethyl-thiadiazole		Sulfaethyl-thiadiazole	Acetyl Sulfaethyl-thiadiazole	
4 5			140	4 4	360	225	4 5
5 0	325	4 6	162	4 7	380	230	5 0
5 5	465	5 2	212	5 2	440	250	5 5
6 0	760	5 6	300	5 6	480	350	6 0
6 5	2,250	6 2	510	6 35	600	650	6 5
7 0	5,900	6 6	740	6 7	1,875	1,140	7 0
7 5	7,300	6 8	1,175	7 1			
8 0	17,000	7 1					
	Sulfamethoxy pyridazine		Acetyl Sulfamethoxy pyridazine		Sulfamethoxy-pyridazine	Acetyl Sulfamethoxy-pyridazine	
4 5	720	4 5	22	4 5	460	165	4 5
5 0	740	4 8	24	5 0	466	168	5 0
5 5	770	5 4	26	5 5	475	174	5 5
6 0	800	5 9	28	6 0	488	182	6 0
6 5	920	6 2	30	6 5	552	212	6 5
7 0	1,380	7 0	41	7 0	862	290	7 0

TABLE II —COMPARATIVE SOLUBILITIES AT 0 5 pH UNIT INCREMENTS OVER THE RANGE 4 5 TO 7 0 IN PHOSPHATE BUFFERS AT 37°

	Mg per 100 ml of Solution					
	4 5	5 0	5 5	6 0	6 5	7 0
Trisulfapyrimidines	96	98	102	109	139	192
Sulfacetamide	1,250	1,590	2,760	5,400	11,300	41,000
Sulfamethylthiadiazole	105	125	200	470	1,820	5,960
Sulfisoxazole	33	45	70	175	510	2,350
Sulfaethylthiadiazole	320	450	660	1,520	4,050	10,800
Sulfamethoxypyridazine	720	740	780	840	1,140	1,380

TABLE III.—COMPARATIVE SOLUBILITIES OF ACETYL SULFONAMIDES AT 0.5 pH UNIT INCREMENTS OVER THE RANGE 4.5 TO 7.0 IN PHOSPHATE BUFFERS AT 37°

Acetyl Derivative	Mg. per 100 ml. of Solution					
	4.5	5.0	5.5	6.0	6.5	7.0
Trisulfapyrimidines	116	121	132	158	230	420
Sulfacetamide	60	125	250	790	1,980	3,900
Sulfamethylthiadiazole	41	50	71	102	260	790
Sulfisoxazole	8	12	38	105	220	710
Sulfaethylthiadiazole	150	190	280	310	620	1,040
Sulfamethoxypyridazine	22	24	26	28	30	41

soxazole 2.35%, sulfamethoxypyridazine 1.38%, and trisulfapyrimidines 0.19%. Sulfaethylthiadiazole exhibited a much higher solubility in phosphate buffer than it did in Mosher's Vehicle, being soluble to the extent of 10.70% in the former at pH 7.0.

The points on the pH scale at which marked acceleration of solubility occurred also varied. In the case of the trisulfapyrimidines a marked increase in solubility commenced at around pH 6.0; with sulfisoxazole, sulfamethylthiadiazole, and sulfacetamide this was evidenced at about pH 5.0 and above and with sulfaethylthiadiazole solubility increased abruptly at pH 5.5 and above. The solubility of sulfamethoxypyridazine remained rather constant over the pH range to about pH 6.0 after which it rose precipitously.

Although the compounds differed markedly in solubility, all curves were characteristically similar with the exception of sulfamethoxypyridazine. This compound was unique in showing somewhat different curve characteristics and in the fact that it was found to be considerably more soluble in phosphate buffer than in Mosher's Vehicle. The acetyl derivative of this compound, however, was similar to the acetyl derivatives of the other compounds in that it was considerably more soluble in Mosher's Vehicle than in the phosphate buffers.

Although the urine of normal individuals is usually considered to have a mean pH of 6.0, it may range from 4.8 to 7.5 (24) in pathological conditions. This pH range is therefore critical in view of the facts here illustrated showing that the solubility of most of the compounds investigated increased 50 to 100-fold from pH 5.0 to pH 7.0. With the trisulfapyrimidines the increase is only three-fold and its combined solubilities are the lowest of the entire group.

Since urinary acidity is increased in conditions where the urine is concentrated, as in fevers (25), solubility is important because as the urine is concentrated, so also is its sulfonamide content, and, as the urinary acidity increases with concentration, the solubility of sulfonamide is markedly decreased, thus producing the possibility of sulfonamide urolithiasis. If normal fluid volume and pH are maintained, it is doubtful if any of the above sulfonamides, with their adequate solubilities, would be dangerous. However, sulfisoxazole and sulfamethoxypyridazine have rather insoluble acetyl derivatives, and it may be that when present in high concentration in acid urine, urolithiasis may be a factor. This would be dependent, however, upon the dose, the rate of excretion, and the extent of acetylation.

SUMMARY

The solubilities of six sulfonamides commonly employed in urinary tract infections and their acetyl derivatives have been investigated over the pH range 4.5 to 7.0 at 37° in phosphate buffers and in a synthetic urine known as Mosher's Vehicle. Of these compounds, sulfacetamide was the most soluble followed, in decreasing order, by sulfaethylthiadiazole, sulfamethylthiadiazole, sulfisoxazole, and trisulfapyrimidines. Sulfamethoxypyridazine was intermediate in the group in that it was not so much affected by pH and was relatively more soluble in the acid region than any of the other compounds with the exception of sulfacetamide. Of the acetyl derivatives, sulfacetamide was also the most soluble at the higher pH levels while at a pH of 5.5 and below, sulfaethylthiadiazole was the more soluble. The combined acetyl derivatives of the trisulfapyrimidines were somewhat more soluble below pH 6.0 than was the acetyl sulfamethylthiadiazole. The latter exceeded the solubility of the former at pH 6.5 and above. Acetyl sulfisoxazole exhibited a rather low solubility at pH levels under 6.5 but increased greatly at higher pH values. Acetyl sulfamethoxypyridazine had a very low solubility over the entire pH range in the buffers.

All of the sulfonamides were somewhat more soluble in Mosher's Vehicle than in the buffers with the exception of sulfamethoxypyridazine which was somewhat more soluble in the phosphate buffers. At pH 6.0 (mean pH of urine) acetyl sulfacetamide was the most soluble of the acetyl derivatives followed by the acetyl derivatives of sulfamethylthiadiazole, sulfamethoxypyridazine, sulfaethylthiadiazole, sulfisoxazole, and trisulfapyrimidines. The acetyl derivatives were also more soluble in Mosher's Vehicle than in the buffer with acetyl sulfamethoxypyridazine being considerably more soluble in Mosher's Vehicle than in the buffer.

The physiological implications of these observations are briefly discussed.

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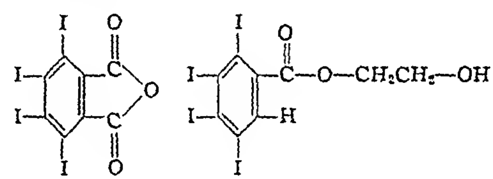
New Iodine Compounds for Hepatolienography*

By ROBERT J. DUMMEL, JAMES D. MADDEN, and SYDNEY F. THOMAS

A new source of radio-opaque compounds for the development of hepatolienography contrast media consists of the class of tetraiodophthalic anhydride derivatives. Hydroxyethyl and hydroxypropyl tetraiodobenzoates were prepared by the decarboxylation of hydroxyethyl and hydroxypropyl tetraiodophthalates. These compounds gave good colloidal dispersions by precipitation in water from acetone. Zirconyl tetraiodophthalate was prepared as a colloidal precipitate, but hydrolysis took place in attempts to neutralize the solvent.

A PERSISTENT SEARCH has been under way to replace thorium dioxide (Thorotrast) as an intravenous colloidal contrast media for the opacification of the liver and spleen, because of the long-term damage to the sites of deposition caused by the permanent radioactive deposits (1). Experiments with tantalum oxide, tungsten oxide (2), zirconium oxide (3), and stannic oxide (4) have been reported. Elimination of the contrast media from the sites of deposition after completion of the diagnosis is desirable. Metal oxides are permanently fixed, but organic iodine compounds offer a possible alternative, if one can be found which will undergo slow metabolic breakdown. Experiments with emulsions of iodinated vegetable oils (Lipiodol) and ethyl esters of iodinated fatty acids (Angiopac and Ethiodol) have been reported (5, 6, 7).

An ideal compound for hepatolienography should be chemically inert, water insoluble, easily dispersed to a stable colloid, and subject to slow elimination from the organism. Among the organic iodine compounds, nearly all soluble injectable contrast media used today are aromatic in structure. We therefore chose as a starting material for a series of possible contrast media the aromatic compound tetraiodophthalic anhydride (1), which contains by weight 77.9 per cent iodine.



I II

The chemically reactive anhydride group can give rise to an open ended series of derivatives such as monoesters, diesters, amides, and imides. Such derivatives should be hydrolyzed metabolically. Information on the chemistry of tetraiodophthalic anhydride was limited to accounts of its synthesis (8), and the formation of the monomethyl and dimethyl esters (9), dioctadecenyl and didodecyl esters (10), several aliphatic imides (11), the anil and phthalein (12).

The results reported below were encountered in attempts to prepare a polyester from ethylene glycol and tetraiodophthalic anhydride by a reaction analogous to the well-known preparation of polymer resins from glycols and phthalic anhydride. Tetraiodophthalic anhydride reacted readily with hot ethylene glycol to give the half-ester, hydroxyethyl tetraiodophthalate. This crystalline solid reverted on dry heating to the anhydride and the glycol. When the half-ester was refluxed in a high-boiling solvent, a resinous, low-melting solid was obtained, which was separated into a crystalline solid and an amorphous resin. The crystalline solid was identified as hydroxyethyl tetraiodobenzoate (II),

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which resulted from the thermal decarboxylation of the half-ester. Precedent for this reaction was found in the reported reactions of tetrachlorophthalic anhydride (13). The presence of polyester in the uncrystallized resin was inferred from hydrolysis to tetraiodophthalic acid, but final proof must depend on molecular weight studies.

The same procedure using tetraiodophthalic anhydride with propylene glycol gave similar products. A crystalline product was identified as hydroxypropyl tetraiodobenzoate.

The low reported toxicity of zirconyl salts (3) and tetraiodophthalate (14) were the basis of attempts to prepare zirconyl tetraiodophthalate.

EXPERIMENTAL

Tetraiodophthalic Anhydride.—The commercially available (Eastman Kodak) product, m. p. 310–322°, was used without further purification. It was found to be readily soluble in dioxane and dimethylformamide, from which recrystallization could be achieved by the addition of chloroform, cyclohexane, or acetic acid. Heating tetraiodophthalic anhydride, alone or in a solvent, above 350° resulted in decomposition with liberation of iodine. Sodium, potassium, and ammonium mono-, and di-salts were prepared by the addition of the calculated amount of base to a dioxane solution of tetraiodophthalic anhydride.

Anal.—A sample was fused with NaOH in a nickel crucible, neutralized, and titrated with standard silver nitrate according to the Volhard method, using tetraiodofluorescein for an indicator. Calcd. for $C_8I_4O_3$: I, 77.88. Found: I, 77.77.

Hydroxyethyl, Hydroxypropyl, and Ethyl Tetraiodophthalates.—Half-esters were readily formed by dissolving the anhydride in refluxing alcohols. Samples of the anhydride were dissolved in ethylene glycol, propylene glycol, and for comparison, in ethanol. In each case, a crystalline yellow solid was obtained. Small samples of each product were heated in a capillary melting point apparatus, with evolution of vapor, and a residue which melted at 205–222°. The melting point was not depressed by mixing the sample with tetraiodophthalic anhydride.

Equivalent Weights.—Calcd. for hydroxyethyl tetraiodophthalate, $C_{10}H_6I_4O_5$: 714. Found: 710. Calcd. for hydroxypropyl tetraiodophthalate, $C_{11}H_8I_4O_5$: 728. Found: 726. Calcd. for ethyl tetraiodophthalate, $C_{10}H_6I_4O_5$: 714. Found: 710.

Hydroxyethyl Tetraiodobenzoate.—Hydroxyethyl tetraiodophthalate (10.8 Gm.), or the equivalent mixture of ethylene glycol and tetraiodophthalic anhydride, was refluxed four hours in dimethylformamide (50 ml.). Evaporation of the solvent under reduced pressure left a dark, opaque residue (17 Gm.). The residue was refluxed with acetone (100 ml.), and the acetone solution was filtered and evaporated to a cloudy amber resin (9.9 Gm.). The cloudy amber resin was refluxed in dichloromethane (100 ml.), and the solution filtered from an amorphous yellow powder. Evaporation of the solvent left a transparent glassy amber resin (8.6 Gm.) which softened to a viscous oil at 90–100°, containing 75.02% iodine. The apparent equivalent weight of 5,520 indicated some unreacted acid. Part of this resin was dissolved in dichloromethane (100 ml.) and extracted with 10% sodium carbonate solution (2 × 100 ml.). The organic phase, considerably lighter in color, was washed with water, dried over calcium chloride, filtered and evaporated to a light yellow oil (4.7 Gm.). Recrystallization from acetone gave a light yellow, semicrystalline solid, m. p. 215–224°.

Anal.—Calcd. for $C_9H_6I_4O_3$: I, 75.59. Found: I, 75.44.

Hydroxypropyl Tetraiodobenzoate.—Starting with hydroxypropyl tetraiodophthalate, or the equivalent mixture of propylene glycol and tetraiodophthalic anhydride, the same procedure as above was repeated to give a similar product. The product was light yellow semicrystalline solid, m. p. 215–222°, and soluble in acetone and dichloromethane.

Anal.—Calcd. for $C_{10}H_8I_4O_3$: I, 74.22. Found: I, 74.32.

Zirconyl Tetraiodophthalate.—The addition of zirconyl chloride (50.2 Gm., 0.153 mole) in water (500 ml.) to a solution of disodium tetraiodophthalate (106 Gm., 0.149 mole) in 1:3 dioxane:water (1,000 ml.) precipitated a pale yellow colloidal dispersion. The precipitate was washed by centrifugation and resuspension, and dried to a pale yellow powder (63 Gm., 0.812 mole, 55% yield).

Anal.—Calcd. for $C_8I_4O_6Zr$: ZrO_2, 15.9. Found: ZrO_2, 16.7. The initial precipitate was stable only in acid solution, and attempts to neutralize the solution with dilute base resulted in hydrolysis to zirconium hydroxide and sodium hydrogen tetraiodophthalate.

Preliminary Dispersion Studies. Hydroxyethyl and hydroxypropyl tetraiodobenzoate were each dissolved in acetone, and the acetone solutions poured into water. In each case, a colloidal dispersion formed with very little gummy precipitation The acetone was removed and the dispersions concentrated by distillation under reduced pressure. Microscopic examination of these dispersions revealed uniformly spherical particles in rapid Brownian motion, with the estimated average particle size of 1 μ. The dispersions settled slowly during several days, and could be partly resuspended by shaking. The half-esters in this series did not give stable dispersions by this technique.

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Metal Chelates and Antitubercular Activity V*

Riboflavin, Riboflavin-5′-phosphate, Alloxan, Phthaloylhydrazine

By WILLIAM O. FOYE, FRED B. BLOCK, and WINTHROP E. LANGE

Metal complexes, which are probably chelates, have been prepared of riboflavin, roboflavin-5′-phosphate, alloxan, and phthaloylhydrazine using divalent copper, cobalt, nickel, iron, and zinc ions. Antitubercular tests in mice showed these chelates to be essentially inactive.

PREVIOUS ATTEMPTS to find a relationship between metal-chelating ability and antituberculotic activity have shown some success. A number of agents which have been employed against human tuberculosis have shown an ability to chelate heavy metal ions (1, 2, 3) and several have been shown to retain activity in the form of heavy metal chelates (4, 5, 6). An example of a class of agents which showed a limited amount of activity in the mouse in the chelated form while the nonchelated agents were inactive has also been found in the case of the *o*-hydroxyazonaphthols (7). In order to inquire further into the possibility that the action of metal chelates against tuberculosis is of fairly general occurrence or whether it is dependent upon limited structural requirements, several other types of metal chelates have been prepared and tested in the mouse. For this purpose, chelates of riboflavin, riboflavin-5′-phosphate, alloxan, and phthaloylhydrazine were used.

METHODS OF PREPARATION

The preparation of metal chelates of riboflavin (8) and of alloxan (9) has already been reported. Chelation of riboflavin-5′-phosphate with nickel and cobaltous chlorides was first carried out at pH 9 using the procedure of Foye and Lange (8) for riboflavin chelates. There was a distinct drop in pH when the alkalinity of the reaction was not maintained, and the isolation of colored, water-insoluble products provided further indication that chelation was taking place. In addition, negative tests for metal ions in the reaction mixture were obtained with ferrocyanide after the reactions were complete. These chelates were insoluble in organic solvents, water, and aqueous alkali and were decomposed by aqueous acid. Since recrystallization was impossible, excess riboflavin-5′-phosphate and sodium ion were removed by Soxhlet extraction using water. Elemental analysis of the dried products revealed an unusually high metal content (see Table II).

In an attempt to prepare chelates having a lower metal content, reactions with several metal ions were carried out at pH 7. Evidence for chelation was again noted by a drop in pH in experiments where the pH was not maintained by alkali. The following drops in pH were noted: with zinc ion, the pH dropped to 4.2, with iron to 5.2, with cobalt to 5.6, with nickel to 5.6, and with copper to 5.8. In the preparation done at pH 7, colored, water-insoluble products were again obtained. Very slight positive tests for metal ion were noted in the reaction mixtures, however, after the reactions were complete. Elemental analysis of these chelates showed in general a ratio of three metal ions to two molecules of riboflavin-5′-phosphate. This amount of metal in the products was still considered somewhat high for animal experimentation, however.

Aqueous mixtures of the products prepared at either pH 7 or pH 9 were then brought to pH 3 to reduce the metal contents still further. Both soluble and insoluble fractions were isolated from this treatment of the cobalt and nickel chelates, but the two fractions from the nickel chelate proved to be identical. The copper and zinc chelates gave only insoluble products, while the iron chelate was unaffected by this treatment. Products having a ratio of two riboflavin-5′-phosphate molecules to one metal ion were obtained from the cobalt, nickel, and zinc chelates, whereas the copper chelate was converted from a ratio of five metal ions to two molecules of riboflavin-5′-phosphate at pH 7 to a ratio of 3:2 at pH 3.

The analytical results of the various riboflavin-5′-phosphate chelates prepared are listed in Table II. The general analytical procedure consisted first of a metal analysis of the product dried *in vacuo* (2–5 mm.) at room temperature. Carbon-hydrogen analyses were then obtained on the products after being dried at a higher vacuum (1 mm.) and the loss of weight observed on this second drying generally corresponded to the theoretical amount for two or three molecules of water. This procedure provided a check on the quantity of water of coordination present, since the Karl Fischer or complete dehydration methods of water analysis failed to give consistent results with these compounds. The color and per cent yield of these products are recorded in Table I.

Chelation reactions of phthaloylhydrazine were carried out with divalent copper, cobalt, and nickel ions at a pH of 9. The insoluble products were purified by washing with water and extraction with acetone. A characterizable ferrous chelate was not obtained. The analytical results indicate a 1:1 ratio of metal ion to phthaloylhydrazine (Table III), but the actual structures probably have a 2:2 ratio. This is

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indicated by the absence of bound water in the copper derivative, where the coordination capacity of four would be satisfied by two molecules of phthaloylhydrazine, and the presence of only two molecules of water for each cobalt or nickel ion, where the usual coordination capacity is six.

BIOLOGICAL ACTIVITY

Antitubercular tests on nonchelated agents of the types under discussion have been reported only for phthaloylhydrazine. Buu-Hoï (10) found this compound to be active against avian tuberculosis in 1946. O- and N-Substituted derivatives of phthaloylhydrazine were later found to be markedly active against human tuberculosis in mouse tests, however (11).

Tests against experimental tuberculosis in mice were carried out at the Lilly Research Laboratories on a number of the compounds discussed above. The copper, cobalt, nickel, iron, zinc, and manganese chelates of riboflavin (8) were essentially inactive when tested at levels of 0.25, 0.1, and 0.02% in the diet. The iron chelate did show slight activity at 0.02%, however. Among the riboflavin-5′-phosphate chelates, the following were tested: the cupric derivative having a 2:3 ratio of ligand to metal, the zinc compound having a 2:1 ratio, the 2:1 cobalt derivative, and the 2·3 ferrous compound. Although these compounds were described as essentially inactive, small increases in survival time were noted at some dietary levels. These results are shown in Table IV.

The 1:1 cobalt chelate of alloxan (9) was tested at dietary levels of 0.01 and 0.05%, and it was found to be toxic at the higher level and inactive at the lower. The cobalt and nickel complexes of phthaloylhydrazine were tested at dietary levels of 0.01 and 0.5%. The cobalt derivative was toxic at the higher level and inactive at the lower, while the nickel derivative was inactive at both levels.

EXPERIMENTAL

Metal Chelates of Riboflavin-5′-phosphate at Various pH's.

pH 9.—Two grams (0.0039 mole) of sodium riboflavin-5′-phosphate (Nutritional Biochemicals Corp.) was added to 50 ml. of water in a beaker equipped with a mechanical stirrer. To the solution was added sufficient 5% sodium hydroxide solution to bring the pH to 9. An aqueous solution of 0.83 Gm. (0.0035 mole) of cobalt (II) chloride hexahydrate or nickel (II) chloride hexahydrate was then added slowly over a period of ten minutes, along with sufficient 5% alkali to maintain the pH at 9. After thirty minutes more of stirring and adjusting the pH, the mixture was centrifuged, and the residue was dried *in vacuo*. The supernatant liquid was made neutral with 5% hydrochloric acid, reduced in volume by evaporation, and treated with ethanol to precipitate excess riboflavin-5′-phosphate. The dried residue was extracted with water in a Soxhlet extractor for twenty-four hours and was then dried *in vacuo* (2–5 mm.) over phosphorus pentoxide for twelve to sixteen hours.

pH 7.—To 50 ml. of water in a beaker equipped with a mechanical stirrer was added 2.0 Gm. (0.0039 mole) of sodium riboflavin-5′-phosphate. To this solution was added sufficient 5% sodium hydroxide solution to bring the pH to 7. An aqueous solution of 0.0039 mole of cobalt (II) chloride hexahydrate, nickel (II) chloride hexahydrate, zinc (II) chloride, ferrous sulfate heptahydrate, or cupric chloride dihydrate was then added slowly over a period of ten minutes, along with sufficient 5% alkali to maintain the pH at 7. After thirty minutes more of stirring and adjusting the pH, the product was isolated and purified as in the preceding procedure.

pH 3.—To 25 ml. of water in a beaker equipped with a mechanical stirrer was added 1.0 Gm. of metal chelate prepared either at pH 7 or 9. The mixture was brought to a pH of 3 by the addition of 5% hydrochloric acid. After one hour of stirring and adjusting the pH, the mixture was filtered and the residue was washed with water and methanol. The product was dried *in vacuo* (2–5 mm.) over phosphorus pentoxide for twelve to sixteen hours.

To the filtrate was added three to four volumes of acetone to precipitate any water-soluble complex. Nearly complete evaporation of the filtrate generally gave the same result as the addition of acetone. The solid was collected, washed with methanol, and dried in the usual manner.

TABLE I.—METAL CHELATES OF RIBOFLAVIN-5′-PHOSPHATE

Formula[a]	Prepared, pH	Color[b]	Yield, %
$R\text{-}Co_6 \cdot 13H_2O$	9	Brown	90
$R_2\text{-}Co_3 \cdot 7H_2O$	7	Yellow-brown	100+
$R_2\text{-}Co \cdot 6H_2O$	3	Dark yellow	77
$R\text{-}Ni_6 \cdot 17H_2O$	9	Gold	97
$R_2\text{-}Ni_3 \cdot 7H_2O$	7	Bright orange	98
$R_2\text{-}Ni \cdot 4H_2O$	3	Gold-orange	81
$R_2\text{-}Zn_3 \cdot 12H_2O$	7	Bright orange	89
$R_2\text{-}Zn \cdot 4H_2O$	3	Gold-orange	100+
$R_2\text{-}Fe_3 \cdot 7H_2O$	7	Rust	90
$R_2\text{-}Fe_3 \cdot 6H_2O$	3	Rust	99
$R_2\text{-}Cu_5 \cdot 13H_2O$	7	Yellow-brown	89
$R_2\text{-}Cu_3 \cdot 4H_2O$	3	Orange	89

[a] $R = C_{17}H_{21}N_4O_9P$ minus two hydrogen atoms for each metal ion in the formula.
[b] Sodium riboflavin-5′-phosphate (Nutritional Biochemicals Corp.) has a gold-orange color.

Metal Chelates of Phthaloylhydrazine

Phthaloylhydrazine (0.01 mole) prepared by the method of Barber and Wragg (12), was dissolved in 50 ml. of water and 5% sodium hydroxide solution to give a pH of 9. An aqueous solution of the metal chloride (copper (II), cobalt (II), or nickel (II)) was then added slowly with stirring, and the pH was maintained at 9. The reaction mixture was stirred for an hour and then allowed to settle overnight. The products were filtered, washed with water, and extracted with acetone in a Soxhlet extractor for three hours. They were dried in air at room temperature. A 72% yield of pale green nickel chelate, a 90% yield of maroon cobalt chelate, and an 82% yield of green copper chelate were obtained, based on the formulas in Table III. It was necessary to centrifuge the reaction mixture to obtain the copper derivative.

Metal Analyses

Cobalt.—A modification of the method of Hillebrand, *et al.* (13), was used. A dilute hydrochloric

TABLE II.—ANALYSES OF THE RIBOFLAVIN-5′-PHOSPHATE CHELATES

Formula[a]	Prepared, pH	Calculated, % C	H	M^{++}	H_2O[c]	Found, %[b] C	H	M^{++}	H_2O[d]
$R\text{-}Co_6 \cdot 13H_2O$	9			34.56	5.24			35.54	5.94
$R\text{-}Co_6 \cdot 10H_2O$		20.88	2.99			20.76	3.36		
$R_2\text{-}Co_3 \cdot 7H_2O$	7			14.62	5.96			14.54	5.93
$R_2\text{-}Co_3 \cdot 3H_2O$		35.90	3.72			35.92	3.81		
$R_2\text{-}Co \cdot 6H_2O$	3			5.47	3.34			5.03	3.20
$R_2\text{-}Co \cdot 4H_2O$		39.20	4.64			39.72	4.75		
$R\text{-}Ni_6 \cdot 17H_2O$	9			31.94	3.27			32.39	2.63
$R\text{-}Ni_6 \cdot 15H_2O$		19.14	3.69			19.10	4.41		
$R_2\text{-}Ni_3 \cdot 7H_2O$	7	33.78	4.17	14.57		34.31	4.68	13.04	
$R_2\text{-}Ni \cdot 4H_2O$	3			5.64	3.46			5.52	3.08
$R_2\text{-}Ni \cdot 2H_2O$		40.61	4.41			40.37	4.83		
$R_2\text{-}Zn_3 \cdot 12H_2O$	7			14.87	2.73			13.99	2.92
$R_2\text{-}Zn_3 \cdot 10H_2O$		31.83	4.40			31.65	3.49		
$R_2\text{-}Zn \cdot 4H_2O$	3			6.24	3.44			9.02	4.26
$R_2\text{-}Zn \cdot 2H_2O$		40.35	4.38			40.16	4.23		
$R_2\text{-}Fe_3 \cdot 7H_2O$	7			13.96	9.01			12.84	8.46
$R_2\text{-}Fe_3 \cdot H_2O$		37.39	3.51			37.68	4.12		
$R_2\text{-}Fe_3 \cdot 6H_2O$	3			14.17	4.57			12.35	4.11
$R_2\text{-}Fe_3 \cdot 3H_2O$		36.19	3.75			36.54	4.08		
$R_2\text{-}Cu_5 \cdot 14H_2O$	7	27.73	4.11	21.57		28.06	3.94	21.69	
$R_2\text{-}Cu_3 \cdot 6H_2O$	3			15.81	2.99			14.60	3.28
$R_2\text{-}Cu_3 \cdot 4H_2O$		34.92	3.79			35.26	4.07		..

[a] $R = C_{17}H_{21}N_4O_9P$ minus two hydrogen atoms for each metal ion in the formula

[b] Carbon and hydrogen analyses were done by the Clark Microanalytical Laboratory, Urbana, Illinois and the Weiler and Strauss Microanalytical Laboratory, Oxford, England. Samples were dried at room temperature *in vacuo* (1 mm.)

[c] This quantity represents the difference in theoretical water content shown by the two consecutive formulas listed under a given pH (e. g., $3H_2O$ for $R—Co_6\ 13H_2O \rightarrow R—Co_6\ 10H_2O$)

[d] This quantity represents the loss of weight on drying at room temperature at 1 mm. pressure the product previously dried at room temperature at 2–5 mm. pressure

TABLE III.—ANALYSES OF THE PHTHALOYLHYDRAZINE CHELATES

Formula	Calculated, % C	H	M^{++}	H_2O	Found, %[a] C	H	M^{++}	H_2O[b]
$C_{16}H_8N_4O_4Co_2 \cdot 4H_2O$	37.67	3.16	22.94	15.2	38.24	3.22	22.71	14.9
$C_{16}H_8N_4O_4Ni_2 \cdot 4H_2O$	37.69	3.18		15.2	37.41	3.33		16.9
$C_{16}H_8N_4O_4Cu_2$	42.95	1.80	28.02	0.0	42.96	2.57	26.95	2.1

[a] Carbon and hydrogen analyses were done by the Weiler and Strauss Microanalytical Laboratory, Oxford, England

[b] The water content was determined by loss of weight on vacuum (2–5 mm.) drying at 100° over phosphorus pentoxide

TABLE IV.—*In Vivo* ANTITUBERCULAR ACTIVITIES OF THE RIBOFLAVIN-5′-PHOSPHATE CHELATES[a]

Compound	In Diet, %	No. of Mice	Mortality, %	Mean Survival Time, Days[b]
$R_2\text{-}Cu_3\ 4H_2O$	0.1	5	60	20.5+
$R_2\text{-}Zn\ 5H_2O$	0.02	5	60	19.8+
$R_2\text{-}Co \cdot 3H_2O$	0.1	5	60	20+
$R_2\text{-}Fe_3 \cdot 6H_2O$	0.2	5	80	19+
INH[c]	0.01	5	0	21+
Controls		10	70	19.7+

[a] Determined at the Lilly Research Laboratories by W. B. Sutton using *M. tuberculosis* H37Rv in mice

[b] A + indicates that the surviving animals were sacrificed at the indicated times for examination of lesions

[c] INH = isonicotinyl hydrazide

acid solution containing 0.03–0.04 Gm. of cobalt per sample was prepared by treating the sample first with 5 ml. of concentrated hydrochloric acid and heating to break the complex, and then adding 195 ml. of water. The solution was heated to about 80°, and approximately 0.5 Gm. of α-nitroso-β-naphthol in 20 ml. of hot, freshly prepared 50% acetic acid was added for every 0.01 Gm. of cobalt present. The precipitate settled, and more reagent was added until it was certain that precipitation was complete. The precipitate was allowed to stand for two hours and was then transferred to a crucible, washed with hot water, and heated slowly to remove carbon. Ignition was carried out in an oxidizing atmosphere at 800–900°, and the residue was weighed as Co_3O_4.

Nickel.—This procedure was modified from that of Hillebrand, *et al.* (14). A solution containing 0.02–0.03 Gm. of nickel was prepared by first treating the complex with 15 ml. of 10% hydrochloric acid and then adding 0.5 Gm. of tartaric acid and 185 ml. of water. It was heated nearly to boiling, sufficient concentrated ammonium hydroxide was added to bring the pH to 7, and it was then made slightly acid with 5% hydrochloric acid. At least 5 ml. of a 1% alcoholic solution of dimethylglyoxime was then added for every 0.01 Gm. of nickel present. Immediately, sufficient dilute (10%) ammonium hydroxide was added dropwise to give a slight excess beyond the point of precipitation. The precipitate was digested on a steam bath for one hour, filtered in a sintered-glass crucible, and washed with hot water. It was dried at 150° for forty-five minutes and weighed as $C_8H_{14}N_4O_4Ni$.

Copper.—A modification of the method of Hillebrand, *et al.* (15), was used. A sample containing from 0.05–0.25 Gm. of copper was treated with 20 ml. of acetic acid to decompose the chelate and then

with 80 ml of water To the solution was added 5 Gm. of potassium iodide dissolved in a little water, and 75 ml. of carbon tetrachloride was added. The mixture was filtered into a separatory funnel, the filtration funnel being washed with carbon tetrachloride, and the filtrate was immediately titrated with thiosulfate solution, preferably having a titer equal to 0.005 Gm of copper. The funnel was shaken after each addition of thiosulfate, the end point was the disappearance of the pink color in the organic layer. The usual method of titration of the aqueous solution could be employed if the chelate did not impart color to the solution

Zinc.—A modification of the method of Kolthoff and Sandell (16) was used A solution containing approximately 0 05–0 1 Gm of zinc was prepared by treating the compound with 3 ml of concentrated hydrochloric acid and then diluting with 200 ml of water. To the solution was added a few drops of methyl red followed by ammonium hydroxide until the solution just turned yellow It was heated to 90°, and 30 ml of 20% diammonium phosphate was slowly added It was kept hot on a steam bath for an additional half-hour, and the precipitate was set aside for two hours The product was filtered through a porcelain crucible, washed with 1% diammonium phosphate until chlorine-free, and then washed with 50% alcohol It was heated slowly until water and ammonia were expelled, and was then heated at 900° to constant weight. It was weighed as $Zn_2P_2O_7$

Iron.—The procedure of Kolthoff and Sandell (17) was used without modification

SUMMARY

1. Metal complexes, which are probably chelates, have been prepared of riboflavin-5'-phosphate and phthaloylhydrazine using divalent copper, cobalt, nickel, iron, and zinc ions.

2. Analytical results on the riboflavin-5'-phosphate chelates show the following general formulas to exist at three pH values: at pH9, $R\text{-}M_6\cdot xH_2O$ for Co and Ni chelates; at pH7, $R_2\text{-}M_3\cdot xH_2O$ for Co, Ni, Fe, and Zn chelates, and $R_2\text{-}M_5\cdot xH_2O$ for Cu; at pH3, $R_2\text{-}M\cdot xH_2O$ for Co, Ni, and Zn chelates, and $R_2\text{-}M_3\cdot xH_2O$ for Cu.

3. Antitubercular tests in mice carried out on a number of these chelates showed them to be essentially inactive, although small increases in survival time were noted with the riboflavin-5'-phosphate chelates.

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The Preparation of Some Derivatives of Oximes*

By LOUIS GASS† and FRANK W. BOPE

Fourteen new esters of a series of oximes have been synthesized. Some of the more important physical properties have been determined.

OXIMES and their derivatives have been found to possess activity as muscle relaxants (1, 2), local anesthetics (3) cholinesterase activators (4), antifungal agents (5, 6, 7), trypanocides (8), antibacterials (9, 10), tuberculostatics (11, 12), and antirickettsials (13). Several oximes have been shown to possess anticonvulsant activity, comparable to diphenylhydantoin, against electric shock (14). However, they also produced certain toxic effects (1, 15–17).

Little work has been done in the field of esters of oximes for the preparation of useful products with the exception of those having insecticidal activity (18). It was, therefore, decided to prepare a series of esters of oximes for later pharmacological testing, having possible anticonvulsant activity similar to the original oximes.

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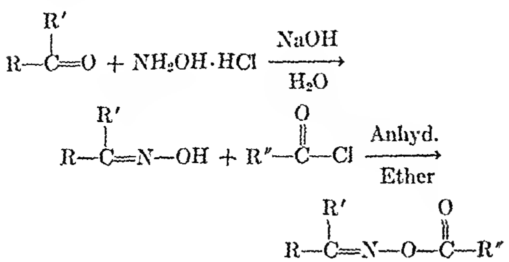
The synthesis of a series of selected esters of five oximes, namely: anisaldoxime, benzaldoxime, acetophenone oxime, benzophenone oxime, and cyclohexanone oxime, is reported herein.

The method of synthesis of these esters was essentially the same as that of a standard esterification except an oxime was used in place of the alcohol. A series of acids in the form of acid chlorides was used to make the esters of each oxime as follows:



Only the syn (α) oximino isomers of the oximes having isomers were prepared since they are more stable than the anti-(β) isomers (19, 20).

EXPERIMENTAL

Synthesis of the Oximes

Cyclohexanone oxime was prepared according to the method of Vogel (21). Recrystallization from petroleum ether resulted in a yield of 80.7% of the oxime melting at 92–93°.

Benzophenone oxime was prepared according to the method described in "Organic Syntheses" (22). The crude product was recrystallized from methanol to give a 78 5% yield of the oxime melting at 144–145°.

syn-Benzaldoxime was prepared by a procedure given by Vogel (23) in a yield of 75 6% with a melting point of 33–34°.

syn-*p*-Methoxy benzaldoxime was prepared by the method used for *syn*-benzaldoxime. The resulting impure product was recrystallized from ethanol in a 75.0% yield melting at 63–64°.

syn-Acetophenone oxime was also prepared by the method used for *syn*-benzaldoxime The crude product was recrystallized from water in a yield of 80 5% and melting at 58–60°.

Synthesis of Esters of the Oximes

The following general procedure was used in preparing all esters of the oximes with the exception of the two listed below where the procedure was modified in order to facilitate purification or crystallization. Analytical data for each compound are listed in Table I.

In a 125-ml Erlenmeyer flask fitted with a magnetic stirrer and cooled in an ice bath was placed 0 05 mole of the oxime and 50 ml. of anhydrous ether to which was added 0 05 mole of the appropriate acid chloride.[1] The mixture was stirred for five minutes after the addition of the acid chloride and the flask was then removed from the ice bath. Upon evaporating the ether under a stream of air, a precipitate formed which was mixed with 200 ml. of a 3% solution of sodium bicarbonate and stirred for ten minutes. The solid material was then filtered with suction, washed with 300 ml. of cold distilled water, and then recrystallized from an ethanol-water mixture after decolorizing with activated charcoal. The recrystallized products were dried *in vacuo* (2 mm. over phosphorus pentoxide) from 40 to 60°.

Cyclohexanone Oxime Phenyl Acetate.—Using the general procedure described above, 5.7 Gm. (0.05 mole) of cyclohexanone oxime was mixed with 7.7 Gm. (0.05 mole) of phenyl acetyl chloride in anhydrous ether. The ether was evaporated under a stream of air and the liquid remaining was dissolved in 100 ml. of benzene. After washing the benzene solution twice with 100-ml. portions of 3% sodium bicarbonate solution, the benzene layer was dried over anhydrous sodium sulfate. This dried benzene solution was decolorized with activated charcoal and the benzene removed under a stream of air. The remaining oil-like product was dried over phosphorus pentoxide in a vacuum desiccator. Several attempts were made to purify the oil-like product by crystallization but without success. When purification was attempted by distillation, the oil-like product distilled at 80°/2 mm. with decomposition.

***syn*-Acetophenone Oxime Phenyl Acetate.**—The same procedure above was used to react 6 8 Gm. (0.05 mole) of *syn*-acetophenone with 7.7 Gm. (0.05 mole) of phenyl acetyl chloride and purify the resulting ester. The oil-like product obtained from benzene was dissolved in 100 ml. of absolute ethanol and decolorized with activated charcoal. The filtrate was placed in a dry ice-acetone bath and distilled water was added slowly with rapid stirring until a precipitate formed. The crystals were filtered with suction and dried in a vacuum desiccator over phosphorus pentoxide in a refrigerator.

Infrared Spectra.—Infrared spectra of the oximino compounds were prepared in the Department of Chemistry of The Ohio State University using a Baird Associates Infrared Recording Spectrophotometer, Model B, equipped with a sodium chloride prism. A potassium bromide pellet of each compound served as the investigated sample since there were no bands present which interfered with the absorption peaks of the oximino compounds. Due to the complexity of the compounds studied, only two characteristic bands were considered; the $-\overset{|}{C}=N-$ band appearing at 6 02 to 6.22 μ (24) and the $-\overset{\overset{O}{\|}}{C}-O-$ band appearing at 5 7 to 5.85 μ.[2] These two characteristic bands were present in the infrared spectra of all the esters prepared.

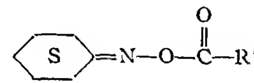
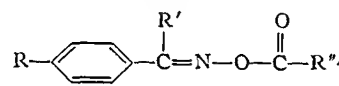
DISCUSSION

Anhydrous ether was used in preference to other solvents for the esterification of the oximes similar to

[1] All acid chlorides were used *per se* with the exception of *p*-nitro benzoyl chloride which was dissolved in anhydrous ether (25 ml.) before adding to the appropriate oxime

[2] Ester carbonyl absorption band was taken from a compilation of known groups by the Ohio State University Department of Chemistry

TABLE I.—ESTERS OF OXIMES

R	R′	R″[c]	Drying[a] Temp °C	Yield, %	M P[b] °C	Molecular Formula	Nitrogen % Calcd	Nitrogen % Found[d]	Saponification Equivalents Calcd	Saponification Equivalents Found No 1	Saponification Equivalents Found No 2
											
		$—C_6H_4—NO_2$	60	95 4	111–112	$C_{13}H_{14}O_4N_2$	10 69	10 86	262 2	266 1	265 1
		$—C_6H_4—OCH_3$	40	81 1	71–72	$C_{14}H_{17}O_3N$	5 67	5 63	247 2	250 1	247 7
		$—CH_2—C_6H_5$		60 6	80°/2 mm[e]	$C_{13}H_{17}O_2N$	6 06	6 01	231 2	231 2	227 3
											
H—	$—C_6H_5$	$—C_6H_4—NO_2$	60	83 9	153–155	$C_{20}H_{14}O_4N_2$	8 09	7 98	346 2	343 9	347 8
H—	$—C_6H_5$	$—C_6H_4—OCH_3$	60	81 0	157–159	$C_{21}H_{17}O_3N$	4 23	4 33	331 2	331 1	338 2
H—	$—C_6H_5$	$—CH_2—C_6H_5$	40	85 1	79–81	$C_{21}H_{17}O_2N$	4 44	4 51	315 2	316 9	317 5
H	—H	$—C_6H_4—NO_2$	60	81 5	166–168	$C_{14}H_{10}O_4N_2$	10 37	10 37	270 1	267 7	275 7
H	—H	$—CH_2—C_6H_5$	40	75 3	70–71	$C_{15}H_{13}O_2N$	5 86	6 02	239 2	233 8	234 4
CH_3O	—H	$—C_6H_4—NO_2$	60	63 3	161–163	$C_{15}H_{12}O_5N_2$	9 33	9 34	300 2	300 0	297 4
CH_3O	—H	$—CH_2—C_6H_5$	40	74 3	55–57	$C_{16}H_{15}O_3N$	5 20	5 32	269 2	264 8	272 2
H	$—CH_3$	$—C_6H_5$	60	83 7	100–102	$C_{15}H_{13}O_2N$	5 86	5 92	239 2	240 8	241 4
H	$—CH_3$	$—C_6H_4—NO_2$	60	77 5	167–169	$C_{15}H_{12}O_4N_2$	9 86	9 86	284 2	280 1	279 5
H—	$—CH_3$	$—C_6H_4—OCH_3$	60	74 3	90–92	$C_{16}H_{15}O_3N$	5 20	5 13	269 2	269 2	270 7
H—	$—CH_3$	$—CH_2—C_6H_5$	5	31 6	166–169	$C_{16}H_{15}O_2N$	5 53	5 35	253 2	253 2	251 8

[a] *In vacuo*, 2 mm , over P_2O_5 [b] Melting points taken on Fisher Johns Apparatus uncorrected. [c] All substitutions on the aromatic rings are in the *p* position. [d] Nitrogen analyses performed by Galbraith Laboratories, Knoxville, Tenn. [e] Boiling point with decomposition. [f] All unsymmetrical compounds were the *syn* (α) forms

the method used by Minunni (25, 26) when it was found that the yield of the final products were higher from this solvent than any other used and that it favored the formation of the *syn*-esters (27, 28) Attempts were made to carry out the esterification of the oximes in dimethylformamide, pyridine, and pyridine-ether mixture With cyclohexanone oxime and benzophenone oxime, the yields of the esters formed ranged from 25 to 40% lower in these solvents than in pure ether. Hauser and Vermillion (29, 30) similarly prepared benzoate esters of oximes and found that the *syn* forms were stable in pyridine alone or in a pyridine-triethyl or propylamine mixture However, in the presence of pyridine and pyridinium chloride, formed during esterification, there was a decomposition of the *syn*-oximes into the anti form and then into the nitrile if the product remained more than a few minutes in this solvent In several cases it was found that esterification of oximes led to the formation of the *syn*- and anti-esters (27, 29), while in another instance esterification was found to take place by the combination of a lactone with an oxime in the presence of triethyl amine (31)

Grammaticakis (32, 33) suggested that N-acylation is favored over O-acylation by showing that acetylation of benzaldoxime yielded the same product as the oxidation of N-acetyl-N-benzylhydroxylamine, namely, N-acetylbenzaldoxime Also absorption spectra studies of acetylated oximes showed the presence of the nitronic structure which favored the N-acetylation This was also confirmed by the reaction of substituted arylaldoximes with the Grignard reagent since the Grignard reacts only with the $-\overset{|}{C}=N-$ group of these compounds The above data substantiated a previous claim of Schmidt (34) that N-acylation took place in preference to O acylation

Exner (35) challenged the above work on the basis of his experimental results with the same types of compounds Ultraviolet spectral analyses which he carried out corresponded to the O-acyl derivatives and the reduction of these O-acyl derivatives with lithium aluminum hydride gave primarily the corresponding alcohol and oxime which was further reduced to the amine

Our infrared analyses substantiate Exner's work They show the $-\overset{|}{C}=N-$ band, as stated previously, appearing in the correct range, 6 02 to 6 22 μ and the $-\overset{\overset{\displaystyle O}{\|}}{C}-O-$ band, 5 7 to 5 85 μ, indicative of an ester linkage This evidence together with the nitrogen analyses favors the existence of O-acylation rather than N-acylation

SUMMARY

Fourteen new esters of a series of oximes, namely: the benzoates, *p*-nitro-benzoates, *p*-methoxy-benzoates and phenyl acetates of cyclohexanone oxime, benzophenone oxime, benzaldoxime, anisaldoxime, and acetophenone oxime are reported.

The esters are characterized by nitrogen analyses, saponification equivalents, and infrared analyses which favor the existence of O-acylation rather than N-acylation.

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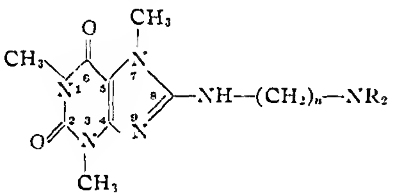
8-(Dialkylaminoalkylamino)-caffeines as Potential Pharmacodynamic Agents*

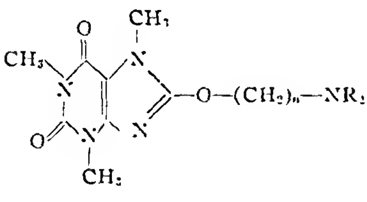
By J. H. BURCKHALTER and DALE R. DILL

A group of caffeine and theophylline derivatives containing basic side chains at position *8* were synthesized as potential pharmacodynamic agents. An attempt was made to explain why type I compounds were at times isolated as the free base and again as the hydrochloride and also why the dihydrochloride and dimethiodide of the same compounds could not be prepared. As an illustration of pharmacodynamic actions, the methiodide of one of the type I compounds caused marked postural hypotension in the absence of autonomic blockade, while no ganglionic blocking or anticholinergic effect was observed.

In a search for chemical structures which might possess hypotensive action, it was considered desirable to attach basic side chains to nuclei which are well known for their pharmacodynamic properties. As a part of these studies, caffeine was chosen since it is known to possess a variety of cardiovascular activities, and, since the reactive 8-chlorocaffeine is readily available (1), it was decided to synthesize a number of 8-dialkylaminoalkylaminocaffeines (I) and 8-dialkylaminoalkoxycaffeines (II). Also



I



II

their quaternary ammonium derivatives were desirable because of the well established activity of quaternary nitrogen compounds against autonomic functions in the body.

The preparative method employed for I was similar to that of Blicke and Godt (2), who made certain 8-alkylaminocaffeines by heating an alcoholic mixture of 8-chlorocaffeine and alkylamines in a pressure bottle. For the purpose of these studies, diamines were used in place of monoamines and most of the results are summarized in Table I together with a number of quaternary ammonium derivatives. Analogous compounds have been made by others by sealed-tube reactions (3).

When ethylenediamine was employed in the reaction designated as Procedure I, 8-(ethylenediamino)-bis-caffeine (V), which contains two caffeine residues, was obtained. When 8-chlorocaffeine was replaced by 8-chlorotheophylline in Procedure I and employed with the appropriate diamine, 8-[4-(1-methylpiperazinyl)]-theophylline (VI) and 8-(N,N-diethylethylenediamino)-theophylline hydrochloride (VII) were synthesized. With N,N-dimethylethylenediamine, 8-chlorotheophylline yielded directly the 8-chlorotheophyllate salt (VIII) instead of the hydrochloride or free base.

During the preparation of this manuscript, a publication appeared which describes the synthesis of three 8-(dialkylaminoalkoxy)-caffeines of structure II (4). For this reason, we wish to report now the synthesis of three compounds of the same general structure.

Although Cooper and Cheney (5) and Chakravarty and Jones (4) were successful in condensing 8-chlorocaffeine with the sodium salts of amino alcohols to obtain type II compounds, we were unsuccessful in employing this approach. Instead, 8-chlorocaffeine, ethylene bromohydrin, and sodium acetate were heated at elevated temperature to give 8-(2-bromoethoxy)-caffeine which was allowed to react with dimethylamine, pyrrolidine, and N-methylpiperazine to produce the expected products (II) in excellent yield.

When N,N-dimethylethylenediamine was employed according to Procedure I, a caffeine derivative of type I (compound 1 of Table I) was obtained in seven different experiments as the free base. This result was expected, especially since a one-hundred per cent excess of starting diamine was employed in order to take up the hydrogen

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TABLE I. 8-(DIALKYLAMINOALKYLAMINO)-CAFFEINES

No	R	Procedure	M. P., °C	Yield, %	Formula	Analyses C Calcd.	Analyses C Found	Analyses H Calcd	Analyses H Found
1	N,N-Dimethylethylenediamino	I	205	89	$C_{12}H_{20}N_6O_3$[a,b]	51 41	51 24	7 19	7 09
2	N,N-Dimethylethylenediamino hydrochloride	I	289	44	$C_{12}H_{20}N_6O_2 \cdot HCl$[a,c,d,e,i]	45 89	45 52	6 69	6 75
3	N,N-Dimethylethylenediamino methiodide	III	285	80	$C_{13}H_{23}N_6O_2I$[n,o,p,r]	36 97	36 78	5 44	6 57
4	N,N-Dimethylethylenediamino *p*-chlorobenzyl chloride	III	236	90	$C_{19}H_{30}N_6O_2Cl_2 \cdot 2H_2O$[l,r,t,u]	47 80	48 12	6 33	6 29
5	N,N-Diethylethylenediamino	I	186	71	$C_{14}H_{24}N_6O_2$[a,b]	54 52	54 28	7 84	7 56
6	N,N-Diethylethylenediamino hydrochloride	I,IIA	270	76,98	$C_{14}H_{24}N_6O_2 \cdot HCl$[a,e,f]	48 75	48 88	7 31	7 55
7	N,N-Diethylethylenediamino ethiodide	III	264	73	$C_{16}H_{24}N_6O_2I$[q,r,s]	41 38	41 30	6 39	6 34
8	N-Methylpiperazinyl hydrochloride	I	344	65	$C_{14}H_{20}N_6O_2 \cdot HCl$[d,g,h]	47 48	47 83	6 44	6 43
9	N,N-Dimethyl-1,3-propanediamino	I	178	61	$C_{14}H_{22}N_6O_2 \cdot H_2O$[g,j,k]	51 46	51 46	7 64	7 68
10	N,N-Dimethyl-1,3-propanediamino methiodide	III	279	90	$C_{14}H_{26}N_6O_2I$[l,m,r]	38 54	38 59	5 78	5 83
11	N,N-Diethyl-1,6-hexanediamino[w]		183	48	$C_{18}H_{32}N_6O_2 \cdot {}^1/_2H_2O$	57 88	58 26	8 97	8 88
12	N,N-Diethyl-1,6-hexanediamino hydrochloride	IIB	236	94	$C_{18}H_{32}N_6O_2 \cdot HCl$[r]	53 92	53 95	8 30	8 32
13	N,N-Diethyl-1,6-hexanediamino ethiodide	III	185	99	$C_{20}H_{37}N_6O_2I \cdot H_2O$[p,v]	44 75	44 61	7 06	7 30

[a] Recrystallized from absolute alcohol three times [b] In one experiment the hydrochloride of this base was also obtained from the reaction [c] Heated at 190–200° [d] Yields may be improved by obtaining second crop crystals [e] Hydrochloride salt of the free base is less soluble in absolute alcohol than is the free base [f] Principal product from reaction [g] Heated at 150°. [h] From 95% alcohol [i] Second crop product may be free base [j] From Skelly Solve B-absolute alcohol mixture [k] Crude product was treated with dilute sodium hydroxide and extracted with chloroform which was then evaporated [l] Heated at reflux for an hour [m] From methyl alcohol [n] Decomposed by light [o] From methyl alcohol or water methyl alcohol [p] Heated at reflux overnight [q] From alcohol-water mixture [r] Washed with ether [s] Refluxed for sixty hours [t] Hydroscopic [u] From alcohol [v] From isopropyl alcohol [w] See experimental

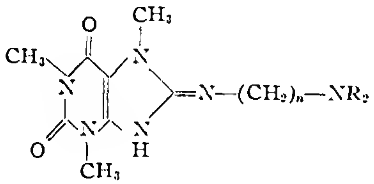
chloride produced. However, *during* one experiment, when the temperature of reaction was elevated to 190–200°, compound *1* was isolated, surprisingly, as the monohydrochloride salt. Further, when N,N-dimethyl-1,3-propanediamine or N-methylpiperazine was used in the same reaction at 150°, the product precipitated in the form of the hydrochloride salt. The last result appears strange in view of the fact that a quantity of the hydrochloride of 8-(N-methylpiperazinyl)-caffeine was insoluble in alcohol at room temperature, but dissolved upon treatment with N-methylpiperazine. Finally, in one experiment involving N,N-diethylethylenediamine, the product was isolated as the free base, while in another experiment under apparently the same conditions the hydrochloride was isolated.

In an attempt to find an explanation for these results, relative pH determinations using the type I bases and the starting diamines in alcoholic solution were made. However, no light was shed thereby upon the results obtained, for, as expected, lower readings were noted for the product than for the starting amine. An explanation based upon chance precipitation was next considered.

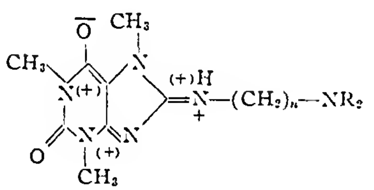
Since a hundred per cent excess of starting amine was employed in the syntheses, at the end of the reaction four compounds should be in equilibrium. They are starting amine, product amine, and their hydrochlorides. Once one of the constituents, for example the product amine, has through chance commenced to separate from solution, the equilibria involving the four compounds would shift to favor removal of hydrogen chloride from the product by the excess of starting amine until a good yield of free base of the product was isolated. Likewise, if the product happened to begin to crystallize as the hydrochloride the equilibria would shift so that the starting amine would give up its hydrogen chloride.

$$\text{Product amine} + \text{starting amine HCl} \rightleftharpoons \text{Product HCl} + \text{starting amine}$$

When an excessive amount of hydrogen chloride was passed into a solution of a type I base (Procedure II), a dihydrochloride salt might have been expected, but only the monohydrochloride resulted. Likewise, attempts to make bis quaternaries (Procedure III) gave the monosubstituted derivatives. Further, neither 8-N-pyrrolidinyl- nor 8-N-piperidinylcaffeine, originally made by Blicke and Godt (2), could be quaternized. These facts suggest that the amino group at position 8 of caffeine derivatives of I is lacking in basicity.



III



IV

Structure III, a tautomeric form of I, does not allow as many resonance structures as I nor does it provide the conjugated system of I. Thus, there would be a large loss in resonance energy in going from structure I to III. For these reasons, only structure I is considered to be of importance, and taking a closer look at it and its resonance forms, one can see the 6-oxo atom as the major perturbation. Because of this carbonyl, one can designate the resonance forms of I as IV. Not only is high resonance energy demonstrated by IV, but also it possesses an excellent conjugated system. If the secondary 8-amino group were to be involved in the formation of a hydrochloride salt, some resonance structures would be prevented and resonance energy would be lowered. Further, it may be observed that I is a vinylogous amide, and, therefore, the ring nitrogen at position 9 as well as the secondary amino at 8 would be lacking in basic properties. Thus, an explanation is offered for the inability of I to form dihydrochloride and dimethiodide salts under the conditions employed.

Pharmacological Results.—After having studied intravenously the action of several of the caffeine derivatives of Table I in anesthetized, vagotomized dogs, Dr. Neil C. Moran, of the National Heart Institute, Bethesda, Md.,[1] made the following statement: "...the compound of most interest is compound 3 because of the marked postural hypotension in the absence of autonomic blockade. Also, compounds 1 and 4 look interesting because of the direct fall in blood pressure in the absence of autonomic blockade. Whether these actions can be attributed to a central nervous system effect can only be determined by further, more definitive experiments."

Dr. Graham Chen, of Parke, Davis and Co. Research Laboratories, Detroit, Mich., found compounds 3 and 8 of Table I, 8-[2-(1-pyrrolidinyl)-ethoxy]-caffeine (IX) and 8-[2-(4-methyl-1-piperazinyl)-ethoxy]-caffeine (X) to be devoid of hypotensive activity when administered orally at 20 mg./Kg. in the perinephritic rat. Also, compounds 3 and X were found to lack hypnotic activity when administered orally at 500 mg./Kg. in the rat. Concerning compound 3, Dr. Chen added, "No ganglionic blocking or anticholinergic effect was observed in pentobarbitalized dogs at a total dose of 10 mg./Kg. intravenously. The intraperitoneal lethal toxicity is about 60 mg./Kg. in mice."

EXPERIMENTAL

8-Chlorocaffeine.—This was prepared by Long's modification of Fischer and Reese's procedure (1). In this method, dry caffeine was dissolved in chloroform and chlorine gas was passed through the solution until the precipitate which first appeared had dissolved. After removal of the chloroform, the desired product in very pure state remained, m. p. 184°. In the present studies, it was found that it was very important to employ anhydrous caffeine. Nearly a quantitative yield of 8-chlorocaffeine was obtained when the commercially available caffeine monohydrate was dried at 60° for three days before use.

8-Chlorotheophylline.—This was prepared by the method of Y. Yoshitomia (6).

N,N-Diethyl-1,6-hexanediamine.—This is the only required diamine which was not readily available from commercial sources. It was prepared from ε-bromocapronitrile and diethylamine by the procedure of Breslow and Hauser (7). The resulting amino nitrile was reduced with Raney nickel to the desired product using the method of Turner (8).

Procedure I.—By a procedure similar to that of Blicke and Godt (2), 0.05 mole of 8-chlorocaffeine or 8-chlorotheophylline was mixed with 0.10 mole of the appropriate diamine and 75 ml. of absolute alcohol in a citrate pressure bottle and heated at 170° for twenty-four hours. After cooling to room

[1] Present address: Emory University, Ga.

temperature, the pressure bottle was opened and the contents were refrigerated overnight. The product was obtained by filtration and frequently purified by recrystallizing from absolute alcohol and washing with anhydrous ether.

Procedure II.—(*A*) The free base of the diamine (0.01 mole) was dissolved in 100 ml. of hot absolute alcohol and, while the solution was still hot, a vigorous stream of dry hydrogen chloride gas was passed into it until it was quite acidic. The volume was then reduced by one half and the mixture was refrigerated. The product was filtered from the solution and then recrystallized from absolute alcohol.

(*B*) The free base of the diamine (0.01 mole) was dissolved in anhydrous ether and a vigorous stream of dry hydrogen chloride gas was passed into the solution. The hydrochloride salt immediately precipitated. The product was collected on a filter and recrystallized from isopropyl alcohol.

Procedure III.—The appropriate diamine (0.01 mole) was dissolved in 75 ml. of boiling anhydrous benzene. Then, the appropriate alkyl halide (0.04 mole) was added and heating was continued as noted in Table I. The mixture was chilled, and the product obtained by filtration was recrystallized and washed with anhydrous ether. The compounds were then dried in the oven at 60° for twenty-four hours. The quaternary salts were frequently unstable to light and hence must be stored in colored bottles to prevent decomposition.

8 - (N,N - Diethyl - 1,6 - hexanediamino) - caffeine (Compound 11, Table I).—To a citrate bottle was added 8.6 Gm. (0.05 mole) of N,N-diethylhexane-1,6-diamine, 12.3 Gm. (0.05 mole) of 8-chlorocaffeine, and 80 ml. of absolute alcohol. After heating at 160° for thirty-four hours, the mixture was chilled. The solid which precipitated was removed by filtration; it proved to be 4.8 Gm. of unreacted 8-chlorocaffeine. The filtrate was evaporated to dryness and the residue was recrystallized twice from isopropyl alcohol. This solid was then mixed and heated on the steam bath with a concentrated sodium hydroxide solution, and extracted with benzene which was then evaporated. The product was purified by three recrystallizations from toluene, washed with Skelly B, and then dried at 50° for forty-eight hours. The yield was 9.0 Gm. (48%), m. p. 182–183°.

8-(Ethylenediamino)-bis-caffeine (V).—This compound was prepared by Procedure I except that equimolar portions of 8-chlorocaffeine (24.6 Gm., 0.1 mole) and ethylenediamine (5.20 Gm., 0.1 mole) were heated together in a citrate bottle at 150° for twelve hours. After cooling, filtering, washing with alcohol, and recrystallizing twice from dimethylformamide, the product was obtained. The yield was 17.3 Gm. (64%), m. p. > 360°.

Anal.—Calcd. for $C_{19}H_{26}N_{10}O_4$: C, 48.64; H, 5.44. Found: C, 49.23; H, 5.39.

8 - [4 - (1 - Methylpiperazinyl)] - theophylline (VI).—Procedure I was used for the preparation of this compound with the substitution of 8-chlorotheophylline for 8-chlorocaffeine. The product was recrystallized three times from dioxane. After drying for twenty-four hours at 60°, a yield of 9.0 Gm. (65%) was obtained, m. p. 275–276°.

Anal.—Calcd. for $C_{12}H_{18}N_6O_2$: C, 51.78; H, 6.52. Found: C, 52.20; H, 6.31.

8 - (N,N - Diethylethylenediamino) - theophylline Hydrochloride (VII).—8-Chlorotheophylline was substituted for 8-chlorocaffeine in Procedure I. The crude product was recrystallized from absolute ethyl alcohol yielding 14.5 Gm. (88%), m. p. 162°.

Anal.—Calcd. for $C_{13}H_{23}ClN_6O_2$: C, 47.19; H, 7.00. Found: C, 47.46; H, 6.92.

8 - (N,N - Dimethylethylenediamino)-theophylline 8-Chlorotheophyllate (VIII).—A mixture of 10.7 Gm. (0.05 mole) of 8-chlorotheophylline, 8.8 Gm. (0.1 mole) of N,N-dimethylethylenediamine and 50 ml. of absolute ethyl alcohol was heated in a citrate bottle at 150° for fifteen hours. After cooling and filtering, the product was recrystallized three times from absolute ethyl alcohol to yield 12.0 Gm. (92%), m. p. 243–244°.

Anal.—Calcd. for $C_{18}H_{25}ClN_{10}O_4$: C, 41.80; H, 5.04. Found: C, 41.80; H, 5.07.

When an alcoholic solution of the product was treated with hydrogen chloride gas, 8-chlorotheophylline was liberated, as indicated by mixed melting point and C-H analysis.

8-(2-Bromoethoxy)-caffeine.—A mixture of 12.3 Gm. (0.05 mole) of 8-chlorocaffeine, 8.2 Gm. (0.1 mole) of sodium acetate and 25 ml. (0.35 mole) of ethylene bromohydrin was placed in a citrate bottle and heated at 190° for forty-eight hours. The contents of the bottle were chilled overnight in the refrigerator. The product was removed by filtration and then purified by three recrystallizations from absolute alcohol. (The filtrate from the reaction may be used with more 8-chlorocaffeine in repeating the experiment.) After decolorizing with charcoal a yield of 14.0 Gm. (83%) was obtained, m. p. 198–199°.

Anal.—Calcd. for $C_{10}H_{13}BrN_4O_3$: C, 37.87; H, 4.13. Found: C, 37.37; H, 3.94.

8-[2-(1-Pyrrolidinyl)-ethoxy]-caffeine (IX).—A mixture of 3.0 Gm. (0.0095 mole) of 8-(β-bromoethoxy)-caffeine, 15 ml. (0.18 mole) of pyrrolidine, and 40 ml. of anhydrous benzene was heated at reflux temperature for twenty hours. After cooling, the product precipitated and was removed by filtration. This solid was extracted with ether. The ether was removed and the residue was recrystallized three times from absolute alcohol and dried at 60° for twenty-four hours yielding 2.8 Gm. (93%) of product, m. p. 189–190°.

Anal.—Calcd. for $C_{14}H_{21}N_5O_2$: C, 54.71; H, 6.89. Found: C, 54.68; H, 6.75.

8 - [2 - (4 - Methyl - 1 - piperazinyl) - ethoxy]-caffeine (X).—To a mixture of 3.0 Gm. (0.0095 mole) of 8-(2-bromoethoxy)-caffeine in 40 ml. of anhydrous benzene, 15 ml. of N-methylpiperazine was added and the solution was heated at reflux temperature for twenty-four hours. After refrigerating for three days, an insoluble material was filtered from the solution. A total volume of 50 ml. of benzene and excess N-methylpiperazine was removed by distillation under reduced pressure. While hot, the residue was mixed with 25 ml. of absolute alcohol. After cooling, crystallization was induced by scratching the side of the beaker with a glass rod. The product was removed by filtration and purified by three recrystallizations from absolute alcohol. It was dried at 60° for twenty-four hours. Second crop crystals were obtained by reducing the volume of the filtrate and cooling to give 3.0 Gm. (91%) of product, m. p. 144–145°.

Anal—Calcd for $C_{15}H_{24}N_6O_3$. C, 53 55, H, 7.19 Found: C, 53 51, H, 6 93

8-(2-Dimethylaminoethoxy)-caffeine (XI).—In a citrate pressure bottle which had been chilled in a sodium chloride ice bath, there was added 2 0 Gm (0.0063 mole) of 8-(2-bromoethoxy)-caffeine and 30 ml of anhydrous dimethylamine which had also been chilled (Bottle should be wrapped as a precaution) The pressure bottle was sealed and the mixture was allowed to stand at room temperature for twenty-four hours After having been chilled to a temperature below 0°, the bottle was unsealed and allowed to stand open to the air for twenty-four hours The solid remaining was recrystallized three times from absolute alcohol and was decolorized once with charcoal. The product thus obtained after drying at 60° for twenty-four hours weighed 1 5 Gm (85%), m. p. 211°.

Anal—Calcd. for $C_{12}H_{19}N_5O_3$: C, 51.23; H, 6 81. Found: C, 51.05; H, 6.55.

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(8) Turner, R A, *ibid*, 68, 1607(1946)

Micromethod for the Estimation of Anesthetics Derived From Aminobenzoic Acid*

Procaine Hydrochloride[1] and Tetracaine Hydrochloride[2] Combined in Cartridges for Dental Anesthesia

By MORRIS E. AUERBACH and MURRAY M. TUCKERMAN†

From a 1-ml. sample, total anesthetic is computed from the ultraviolet absorption of the extracted anesthetic bases in strongly acid solution. The minor constituent, tetracaine hydrochloride, is determined by simultaneously diazotizing the procaine and nitrosating the tetracaine. A salt, soluble in water, is produced from the procaine diazonium chloride by coupling it quantitatively with Chicago acid. The nitroso-tetracaine is then extracted from the alkaline medium with chloroform and determined spectrophotometrically.

In 1957 Pratt (1) reported a spectrophotometric titration with nitrous acid for the estimation of procaine hydrochloride, propoxycaine hydrochloride, tetracaine hydrochloride, and the combination of procaine hydrochloride with tetracaine hydrochloride. For the commercially available formula[3] containing 20.0 mg. procaine hydrochloride and 1.5 mg. tetracaine hydrochloride per ml., Pratt required a 20-ml. sample. The method presented below requires a 1-ml. sample of the same formulation. Since this formulation is packed in 2-ml. doses, the method allows the estimation of the anesthetics in the individual cartridges.

PROCEDURE

Reagents and Apparatus.—Sodium carbonate solution, 10%; chloroform, alcoholic hydrogen chloride, 7 *M* (prepared by absorbing hydrogen chloride gas in alcohol); sulfuric acid, 7 *N*; sodium nitrite, 7%; hydrochloric acid, 1 *N*; Chicago acid (1-amino-8-naphthol-2,4-disodium sulfonate) recrystallized by the method of English (2), 2%

Apparatus includes: Beckman DU spectrophotometer or equivalent

Extraction of Anesthetic Bases.—Transfer a 1 00-ml sample to a separatory funnel containing 20 ml of water Add 2 ml of 10% sodium carbonate solution, mix Extract with three 25-ml. portions of chloroform, pooling the chloroform extracts in a separatory funnel Wash the pooled chloroform extracts with 15 ml of water Filter the washed chloroform extracts through creped filter paper into an evaporating dish, add 0 2 ml of 7 *M* alcoholic hydrogen chloride, evaporate to dryness Take up residue in water to make 100 0 ml

This extraction separates the anesthetic bases from their decomposition products, from inorganic salts, and from phenolic bacteriostats and preservatives

Total Anesthetic.—Transfer a 25-ml. aliquot of the solution of extracted anesthetic bases to a 50-ml volumetric flask, dilute to the mark with 7 *N* sulfuric acid, mix well Determine the absorbance of the solution in a spectrophotometer at its absorption maximum about 272 mμ, using 25 0 ml of 7 *N* sulfuric acid diluted to 50 0 ml. with water as a blank

* Received August 9, 1958, from the Sterling Winthrop Research Institute, Rensselaer, N Y
† Present address Temple University, School of Pharmacy, Philadelphia 40, Pa
[1] Trademark Novocain (Winthrop Laboratories)
[2] Trademark, Pontocaine Hydrochloride (Winthrop Laboratories)
[3] Novocain Pontocaine Cobefrin (N P C) Solution, available from the Cook-Waite Laboratories, 1450 Broadway, New York, N Y

$$\% \text{ Total Anesthetic} = 200\, A_{max} / E^{1\%}_{1\,cm} \text{ for procaine hydrochloride}$$ (A_{max} is the measured absorbance at the wavelength of maximum absorption)

With Beckman DU No. 61607, the absorption maximum for procaine hydrochloride is at 272 mμ with $E^{1\%}_{1\,cm}$ = 35.6 in sulfuric acid ranging in concentration from 2 N to 7 N. In the same instrument, the absorption maximum for tetracaine hydrochloride is at 272 mμ with $E^{1\%}_{1\,cm}$ = 34.5 in sulfuric acid ranging in concentration from 2 N to 7 N.

The absorbance of the sulfuric acid blank read against distilled water at 272 mμ is 0.000.

Obviously the recommended method for calculating total anesthetic introduces an error, since it ignores the fact that the absorbance of tetracaine hydrochloride is a little lower than that of procaine hydrochloride. However, for the commercial ratio of drugs, the error involved is not important. Calculation will show that for a solution actually containing 0.2150% total anesthetic, the experimental value (assuming no other errors), will be 0.2145%. The deliberate error therefore amounts to 5 parts in 2,150.

Tetracaine Hydrochloride.—Transfer a 50-ml. aliquot of the solution of extracted anesthetic bases to a separatory funnel, add 1 ml. of 7% sodium nitrite, mix well, add 1 ml. of 1 N hydrochloric acid, mix well, let stand for five minutes. Add 2 ml. of 10% sodium carbonate solution, mix, add 2 ml. of 1% Chicago acid, mix, let stand for one minute. Extract with three 25-ml. portions of chloroform, pooling the chloroform extracts in a separator. Wash the pooled chloroform extracts with 15 ml. of water. *Filter the washed chloroform extracts* through creped filter paper into a 100-ml. volumetric flask, dilute to the mark with chloroform, mix well. Determine the absorbance of the chloroform solution in a spectrophotometer at its absorption maximum (about 290 mμ) against a blank prepared by treating about 10 mg. of procaine hydrochloride in 50.0 ml. of water as described above.

$$\% \text{ Tetracaine Hydrochloride} = 200\, A_{max} / E^{1\%}_{1\,cm} \text{ for tetracaine hydrochloride after extraction and nitrosation}$$

One mg. of tetracaine hydrochloride, nitrosated, extracted with chloroform, and the extract diluted to 100.0 ml. had, in the Beckman DU No. 61607, an absorption maximum at 290 mμ. Six replicates had measured absorbances of 0.458, 0.455, 0.454, 0.456, 0.452, 0.455, average = 0.455, standard deviation by the method of Dean and Dixon (3) ± 0.002.

The absorbances of six blanks read against chloroform at 290 mμ were 0.026, 0.028, 0.024, 0.024, 0.026, 0.026, average = 0.026, standard deviation ± 0.002.

Procaine Hydrochloride.—Subtract the per cent of tetracaine hydrochloride found from the per cent of total anesthetic found to obtain per cent of procaine hydrochloride.

If the solution is expected to contain a ratio of tetracaine to procaine hydrochloride of greater than 0.30 Gm. to 2.00 Gm., a more accurate calculation of procaine hydrochloride may be made as follows. Determine the absorbance at about 272 mμ as directed for Total Anesthetic, then use the following form for computation:

$$\% \text{ Procaine Hydrochloride} = [200\, A_{max} - (\% \text{ Tetracaine HCl} \times E^{1\%}_{1\,cm} \text{ tetracaine HCl})]/E^{1\%}_{1\,cm} \text{ procaine HCl}$$

PRECISION AND ACCURACY

Six replicates of a solution made up to contain 20.000 mg. of procaine hydrochloride and 1.500 mg. of tetracaine hydrochloride per ml. were assayed with the results shown in Table I.

As an additional check of the method, aliquots of a commercial sample, deliberately partially decomposed by heating, were assayed by Mr. Pratt by his spectrophotometric nitrous acid titration, using a 20-ml. sample, whereas a 1-ml. sample was used in the method presented here. Results are given in Table II.

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Average	2.14	0.150
Standard deviation	±0.01	±0.001

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	Procaine Hydrochloride Found, %	Tetracaine Hydrochloride Found, %
Spectrophotometric titration	2.06	0.125
Micromethod	2.03	0.129

DISCUSSION

The qualitative and quantitative changes in the ultraviolet absorption spectra of aromatic amines as pH of the solvent changed over the range of 2 to 12 have been noted by many authors. As early as 1950, however, the marked change in absorption spectrum of procaine as the acidity of the aqueous medium was increased from 0.01 N to 7 N was recognized in this laboratory.[4] This change in spectrum at high acid concentrations was found to be a property common to all p-aminobenzoic acid derivatives investigated. In neutral medium, these compounds have an absorption maximum at 300 ± 10 mμ, but in solutions of acidity greater than 1 N all except propoxycaine have an absorption maximum at 272 ± 1 mμ. Propoxycaine, which differs from the other p-aminobenzoic acid anesthetics in having a propoxy group substituted on the benzene ring, has an absorption spectrum strikingly different from the other members of the group. It shows two absorption maxima

[4] To an important extent, the work of H. Wm. Eckert

Anal.—Calcd. for $C_{15}H_{24}N_6O_3$: C, 53.55; H, 7.19. Found: C, 53.51; H, 6.93.

8-(2-Dimethylaminoethoxy)-caffeine (XI).—In a citrate pressure bottle which had been chilled in a sodium chloride ice bath, there was added 2.0 Gm. (0.0063 mole) of 8-(2-bromoethoxy)-caffeine and 30 ml. of anhydrous dimethylamine which had also been chilled. (Bottle should be wrapped as a precaution.) The pressure bottle was sealed and the mixture was allowed to stand at room temperature for twenty-four hours. After having been chilled to a temperature below 0°, the bottle was unsealed and allowed to stand open to the air for twenty-four hours. The solid remaining was recrystallized three times from absolute alcohol and was decolorized once with charcoal. The product thus obtained after drying at 60° for twenty-four hours weighed 1.5 Gm. (85%), m. p. 211°.

Anal.—Calcd. for $C_{12}H_{19}N_5O_3$: C, 51.23; H, 6.81. Found: C, 51.05; H, 6.55.

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(4) Chakravarty, D. C., and Jones, J. W., THIS JOURNAL, 47, 233(1958)
(5) Cooper, D. E., and Cheney, L. C., U. S. Pat. 2,688,618, 1954
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in neutral solution (at 280 and 303 mμ) and two in strongly acid solution (at 235 and 297 mμ)

In the assay of combinations of anesthetics derived from *p* aminobenzoic acid and containing tetracaine, it should be noted that the chief chemical distinction between tetracaine and the anesthetics related to it is that tetracaine is a secondary aromatic amine, whereas the others are primary aromatic amines This difference in structure is responsible for a difference in behavior toward nitrous acid The primary amines react quickly and quantitatively to form diazonium salts, tetracaine reacts more slowly, but still quantitatively, to form the N-nitroso derivative

The stability of N-nitroso compounds shows wide variation N Nitroso tetracaine is stable enough for use in the technique described, that is, in aqueous carbonate solution and in chloroform

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(2) English F I *Anal Chem* 19, 458(1947)
(3) Dean R B and Dixon W J *ibid*, 23, 636(1951)

Book Notices

The Health of a Nation (Harvey W Wiley and the Fight for Pure Food) By OSCAR E ANDERSON, JR The University of Chicago Press, Chicago, 1958 ix + 333 pp 15 5 x 23 5 cm Price $6

This book covers the history of the long fight for progressive food legislation which brought about the Federal Food and Drugs Law of 1906, and of the continuing struggle which resulted in attempting to administer its provisions The main emphasis, quite properly, is on the life and accomplishments of Dr Harvey W Wiley, who, as chief of the Department of Agriculture's Bureau of Chemistry, marshaled the forces for federal regulation of the food and drug industries, and later served as chief administrative officer of the 1906 Food and Drugs Act

Proceedings of the National Conference on Hospital Acquired Staphylococcal Disease Sponsored by U S Public Health Service—Communicable Disease Center—National Academy of Sciences—National Research Council U S Dept of Health, Educ, and Welfare, Atlanta, 1958 xi + 178 + xxv pp 20 5 x 26 5 cm

Papers and discussions presented during a national conference on hospital acquired staphylococal disease

Poliomyelitis Compiled and Edited for the International Poliomyelitis Congress J B Lippincott Co, Philadelphia, 1958 xviii + 684 pp 17 5 x 25 5 cm Price $7 50

Papers and discussions presented at the Fourth International Poliomyelitis Conference

Catalysis Vol VI Edited by PAUL H EMMETT Reinhold Publishing Corp, New York, 1958 vi + 706 pp 15 x 23 cm Price $19 50

This sixth volume covers catalytic cracking, isomerization of hydrocarbons, polymerization, polymerization of olefins, alkylation, and hydro reforming

This book, together with the other volumes of the series, should prove of tremendous value to physical chemists and to every industry in which catalysis is involved

Volkstumliche Namen der Arzneimittel, Drogen, Heilkrauter und Chemikalien Vierzehnte Auflage Edited by JOHANNES ARENDS Springer Verlag, Berlin, Gottingen, Heidelberg, 1958 v + 411 pp 13 x 18 5 cm Price DM 15

A dictionary of German vernacular drug names for older botanicals and medicinal chemicals

Biological Laboratory Data By L J HALE John Wiley & Sons, Inc, New York, 1958 x + 132 pp 12 x 18 5 cm Price $2 75

A handbook of mathematical, physical, chemical, statistical, and other data used frequently by biologists

Essentials of Therapeutic Nutrition By SOLOMON GARB Springer Publishing Co, Inc, New York, 1958 ix + 147 pp 14 x 21 cm Price $2

An outline of the principles of nutrition, therapeutic diets, and reference tables designed especially for nurses

A History of the American Soft Drink Industry By JOHN J RILEY American Bottlers of Carbonated Beverages, Washington, D C, 1958 xii + 302 pp 15 x 22 5 cm Price $10

Outlines early efforts in Europe during the 1700's to duplicate the effervescent waters of the natural springs, and traces initial European developments The interest in the United States in the same subject since 1800 is also described, followed by the origin of its commercial aspects when the artificially carbonated waters were sold primarily in drugstores The part played by pharmacists in the early development of the soft drink industry in this country is emphasized throughout

Social Aspects of Psychiatry Edited by BENJAMIN PASAMANICK and PETER H KNAPP Psychiatric Research Reports, American Psychiatric Assoc, Washington 9, D C, 1958 208 pp 15 x 23 cm Paperbound Price $2

Consists of a series of papers presented to the Regional Research Conference held at Columbus, Ohio, on February 24–25, 1958

CORRECTION

Change the name of the author of "Phosphorus and Its Compounds" in the Book Notices section [THIS JOURNAL, 48, 133(1959)] to read John R Van Wazer instead of John R Van Waxer

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII APRIL 1959 NUMBER 4

The Colorimetric Determination of Certain Local Anesthetics With Sodium 1,2-Naphthoquinone-4-sulfonate*†

By EDWARD G. FELDMANN

The quantitative reaction between sodium 1,2-naphthoquinone-4-sulfonate and primary amines has been applied to the assay of certain local anesthetic compounds. The improved colorimetric method which is employed is particularly useful since it is simple and highly selective, and yet provides a relatively high degree of precision and accuracy. While the presence of secondary amines causes interference with most other chemical methods of assay, neither secondary nor tertiary amines display any adverse effect in the procedure described. Specific application to a commercial dosage form is discussed.

LOCAL ANESTHETICS, because of their basic amino groups, have been conveniently assayed for many years by the use of acid-base titration methods. More recently, instrumental methods and particularly ultraviolet spectrophotometry have provided additional and often improved methods of analysis for these compounds. Instrumental methods are especially useful when the anesthetic agents are found incorporated in various dosage forms. However, certain dosage forms on the commercial market include various other ingredients which seriously complicate these conventional methods of analysis.

Any material displaying acidic or basic properties is potentially capable of interference with an acid-base titration. Often these materials are difficult to extract or otherwise remove for this same reason. Similarly, compounds which display absorption, particularly in the long wavelength regions of the ultraviolet spectrum, will cause interference in simple ultraviolet methods of assay. For these reasons, a more selective method for the determination of certain local anesthetic compounds was sought. The classical Folin method for the determination of amino acids in blood (1) provides a simple, direct, and relatively accurate method for the determination of compounds containing a primary amine group. While this procedure has been modified by several investigators (2–7), it has received little attention as a method for the determination of other primary amino compounds which might be present in other systems. Vonesch and Guagnini (8) have employed one modification of this method for the determination of local anesthetics. The specific procedure employed by these investigators, however, suffers in several respects: (*a*) while the color producing reaction proceeds most quantitatively under slightly alkaline conditions (9), no provision is made to adjust the pH of the reaction; (*b*) bleaching the excess reagent with strong alkali following the reaction gives rise to interfering reaction products; (*c*) since the colored complex produced by many local anesthetics is insoluble in dilute alkali except at very low concentrations, the determination of many local anesthetics is seriously limited; and (*d*) the resulting relationship between con-

* Received September 20, 1958, from the Division of Chemistry, American Dental Association, Chicago, Ill

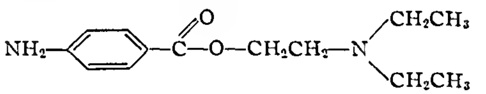
† Analysis of Local Anesthetics II For the preceding paper in this series see THIS JOURNAL, 47, 676(1958)

The author wishes to express his appreciation to Dr J Roy Doty and Mr Henry M Koehler for their many helpful comments, and to Mrs Helen Jones for her assistance in preparing the manuscript.

centration and color intensity is somewhat nonlinear.

Furman and co-workers (7) have reported a greatly improved modification of the Folin procedure and have employed it with good success in the determination of amino acids. This method, when employed in the determination of local anesthetics, was found to be highly satisfactory in this laboratory. The procedure has none of the disadvantages of the Vonesch and Guagnini method which have been noted above. The Furman modification was found to give a reproducible straight-line relationship between color development and concentration for those local anesthetics containing primary amino groups. Moreover, other anesthetics having secondary or tertiary amino groups, but no primary amines, produced no color, and therefore gave no interference in the procedure. As a result, this method provides a means of assaying selectively for one anesthetic agent containing a primary amine in the presence of a second anesthetic having only secondary and tertiary amino groups.

One such dosage form mixture is currently being marketed in dentistry. This product[1] contains both 2′-diethylaminoethyl 4-aminobenzoate hydrochloride (procaine hydrochloride) and 2′-dimethylaminoethyl 4-butylaminobenzoate hydrochloride (tetracaine hydrochloride). The structural formulas for procaine (I) and for tetracaine (II) are illustrated. Because of the



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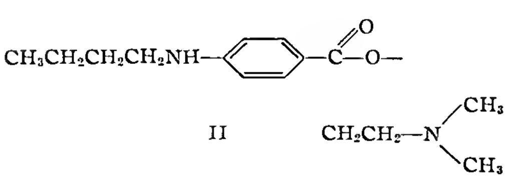

II

very similar chemical and physical properties of these two drugs, they cannot be assayed directly by acid-base titration, nor can they be conveniently quantitatively separated. Moreover, their ultraviolet properties are such that they give rise to mutual absorption interference (see Fig. 1) and as a result, direct determination is difficult and subject to relatively large errors. The assay procedure described herein, however, enables the direct determination of micro quantities of the primary amine procaine, without interference from the presence of the nonprimary amine tetracaine. Pratt (10) recently described a procedure using a nitrous acid titration for the determination of procaine-tetracaine mixtures. His procedure, however, suffers from the need of a large sample, and from the fact that a specially constructed titration chamber is required.

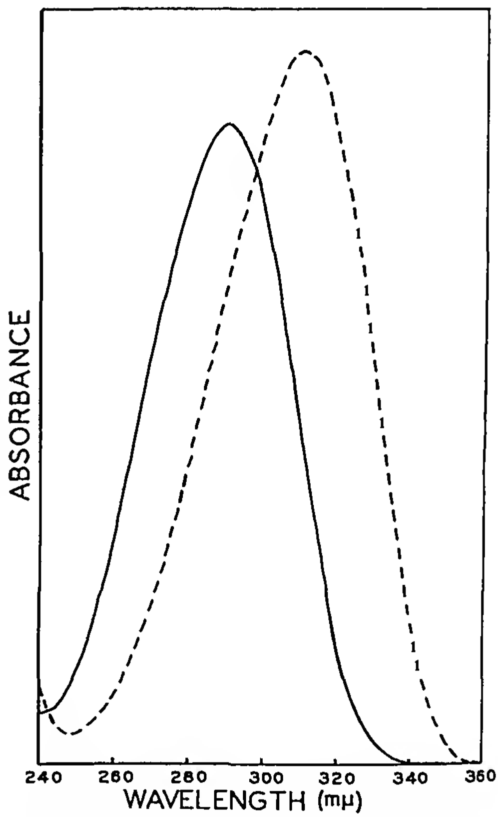

Fig. 1.—Ultraviolet absorbance curves of aqueous solutions; —— procaine hydrochloride; – – – – tetracaine hydrochloride; each 0.01 mg. per ml.

EXPERIMENTAL

Chemicals.—Samples of the local anesthetics employed were generously supplied by their manufacturers: tetracaine hydrochloride and propoxycaine hydrochloride by Cook-Waite Laboratories, Inc., Subsidiary of Sterling Drug; butethamine hydrochloride, metabutethamine hydrochloride, metabutoxycaine hydrochloride, and naepaine hydrochloride by Novocol Chemical Manufacturing Co., Inc.; butacaine sulfate, ethyl aminobenzoate, and procaine hydrochloride by Abbott Laboratories; lidocaine by Astra Pharmaceutical Products, Inc.: meprylcaine hydrochloride by Oradent Chemical Co. Inc.; and piperocaine hydrochloride by Eli Lilly and Co. Sodium 1,2-naphthoquinone-4-sulfonate (Eastman Kodak white label) was employed without fur-

[1] Cook-Waite Laboratories, Inc., markets a solution for injection containing 2.0% procaine hydrochloride (Novocain) and 0.15% tetracaine hydrochloride (Pontocaine Hydrochloride).

ther purification. All other chemicals used were the highest grade of the commercially available materials.

Apparatus.—A Beckman Model DK-2 recording spectrophotometer, with silica cells of 1.00 cm. light path, was employed to record the absorption spectra. Absorbance values at peak wavelengths were checked using a Beckman DU spectrophotometer. All other absorption measurements, including those for the preparation of standard curves, were made employing a Klett-Summerson colorimeter with a No. 50 filter.

Procedure.—A sample of the unknown solution and graded aliquots of a stock standard solution, containing a maximum of 3×10^{-6} mole of primary amine, are transferred to 50-ml. volumetric flasks. Each solution is diluted to approximately 20 ml. with water, and exactly 3 ml. of hydrochloric acid (0.050 *N*) and 3 ml. of sodium carbonate (0.114 *N*) are added. The solutions are mixed, 1 ml. of 1.0% sodium 1,2-naphthoquinone-4-sulfonate solution (prepared fresh each day) is added, the solutions are again mixed, and are allowed to stand in the dark for one and one-half to two hours. Excess reagent is then bleached by the addition of 1 ml. of acetate buffer (25 Gm. sodium acetate trihydrate and 250 ml. glacial acetic acid per liter) and 3 ml. of 4% sodium thiosulfate solution. The solutions are then made up to volume and mixed. Colorimeter readings are taken between ten and twenty minutes from the time that the thiosulfate solution is introduced.

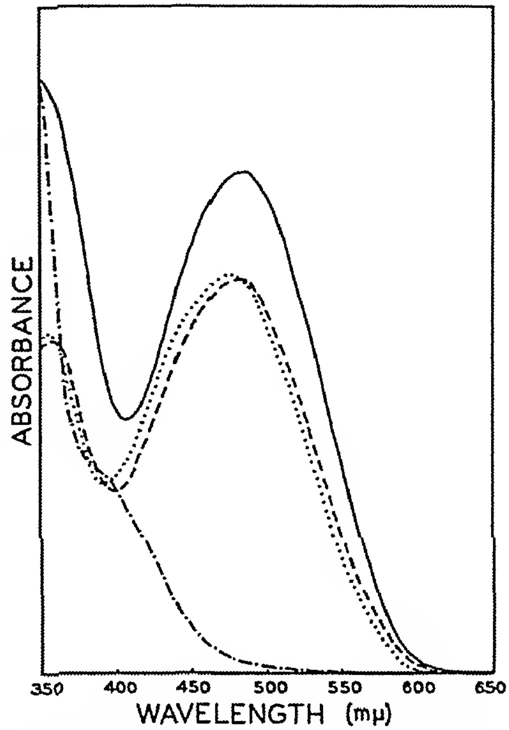

Fig. 2.—Visible absorbance curves of color complexes; —— procaine complex; – – – – metabutethamine complex; . . . . metabutoxycaine complex; –·–·–· reagent blank.

RESULTS AND DISCUSSION

Reaction Involved and Influence of Chemical Structure.—In this assay procedure the yellow sodium 1,2-naphthoquinone-4-sulfonate, in the presence of alkali, reacts with a primary amine-containing compound to yield a highly colored red or orange-red product. Chemically this colored product is an imine resulting from the displacement of the sulfonate radical (7, 9). The excess yellow reagent is then bleached with sodium thiosulfate after making the solution slightly acid with acetate buffer. The absorption curve in the visible spectrum for a typical sample of procaine derivative (after bleaching) is shown in Fig. 2. It will be noted that the absorption maximum occurs at 483 mμ. Reagent blank readings at this wavelength are very low.

In comparing absorption curves of the colored solutions obtained with equimolar concentrations of the various local anesthetics, it was noted that the curves were almost identical for structurally similar local anesthetics. Compounds such as butethamine, propoxycaine, butacaine, and naepaine all produce color which absorbs at the same wavelength maximum and with essentially the same intensity as does procaine. Structurally all of these compounds have an aromatic primary amino group in the position *para* to the carboxylic ester linkage. Metabutethamine and metabutoxycaine, both of which contain an aromatic primary amine in the position *meta* to the ester linkage, show somewhat different absorption characteristics (see Fig. 2). This is in keeping with the findings reported (7) for the amino acids; this difference is attributed to the character of the inductive effect caused by the R group of the amine. It can be seen from the figure that the absorption curves for these *meta* amino compounds are less intense and the maxima occur at somewhat lower wavelengths (about 478 mμ).

The local anesthetics meprylcaine, tetracaine, piperocaine, and lidocaine, each of which has a secondary or tertiary amine group but no primary amine, give no color formation with this procedure.

For the preparation of standard curves, or for routine assay procedures, the color intensity can be conveniently measured by employing a simple filter colorimeter in the region of 500 mμ. A series of such standard curves is presented in Fig. 3. These curves were prepared by plotting observed colorimeter readings *versus* the volumes taken of equimolar concentrations of the various anesthetics. It should be noted that in all cases the ratio of anesthetic concentration to colorimeter reading follows a straight-line relationship; that is, Beer's law holds for this system. It can also be seen that the differences previously noted for the *meta* amines are again in evidence.

Color Development and Stability.—The assay method is essentially a micro procedure, and a maximum of 3×10^{-6} mole of the local anesthetic being determined can be present in the sample. Hence, a maximum of 0.04 ml. of commercial solution containing 2% procaine hydrochloride can be employed as the sample, while 0.02 ml. is preferable. Since the volume of the sample employed can be as great as 20 ml., solutions can be assayed which contain as little as 1.5×10^{-8} mole per ml.; that is, about 3 μg. of local anesthetic per ml. In the course of this investigation it was found that if quantities of the amine larger than 3×10^{-6} mole were present in

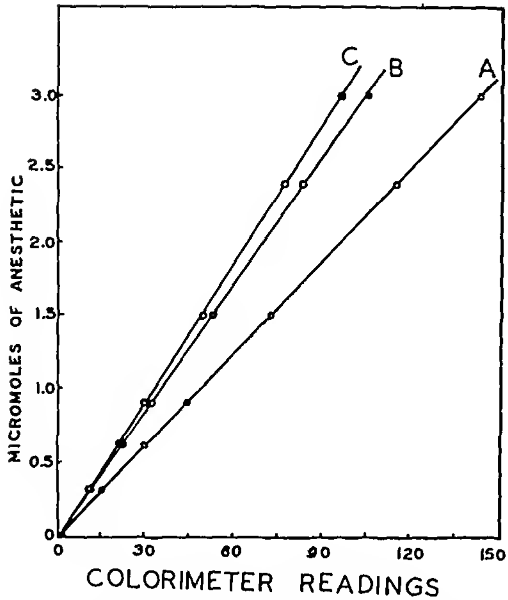

Fig. 3.—Standard curves prepared by plotting anesthetic concentrations *versus* colorimeter readings, A—procaine complex, B—metabutethamine complex, C—metabutoxycaine complex

the sample, readings which were lower than theoretical resulted When the concentration of each of the reagents was then doubled, greater color development occurred with the large amine samples, but the colored complex often partially precipitated from solution By increasing the concentration of only the color developing reagents, and employing normal concentrations of the bleaching reagents, dye precipitation was avoided However, instrument readings indicated that the color development was no longer as closely proportional to the local anesthetic concentration as had been previously observed when normal concentrations of all of the reagents were employed

Previous investigators, in discussing the use of sodium 1,2-naphthoquinone-4-sulfonate in the assay of amino acids, have mentioned various time intervals for maximum color development, and little regarding the duration of color stability after bleaching Folin (1) suggested that the color should be allowed to develop for nineteen to thirty hours; Furman (7) recommended two and one-half hours; and Sahyun (3) suggested ten minutes at 100° None, however, commented on the stability of the color following bleaching In this study it was found that results were most reproducible when the color was allowed to develop for one and one half to two hours at room temperature, and the color intensity was determined within ten to twenty minutes from the time that the thiosulfate was introduced The shorter optimum reaction time here might reflect a greater reactivity of the aromatic amines as compared with the aliphatic amines present in the amino acids Reading color intensity less than five or ten minutes after addition of the bleaching reagents seems to introduce some slight error due to incomplete bleaching, while readings after twenty minutes are somewhat low due to a gradual fading of the color. Colorimeter readings taken from forty-five to sixty minutes after the addition of thiosulfate were generally about 5% lower than readings taken within twenty minutes. Repeated readings on a series of different samples indicated good color stability for approximately thirty minutes, after which very gradual fading occurs over several hours time.

In the case of several anesthetics, particularly butacaine, naepaine, and ethyl aminobenzoate, it was noted that the solutions were quite turbid after color development, but prior to buffering and bleaching This was attributed to insolubility of the color complex in alkaline solution, since addition of the acidic acetate buffer, in every case except for ethyl aminobenzoate, served to immediately clarify the solution Ethyl aminobenzoate, in contrast to the other local anesthetics, does not contain a second amine which is capable of being salted, and therefore solubilization of its complex by acid would not be expected. Only in very dilute concentrations does the ethyl aminobenzoate complex not precipitate, and in these concentrations color formation follows the same curve as that of the other *p*-aminobenzoate anesthetics

Precision and Accuracy.—Some indication of the precision given by the method can be seen by examination of Table I

TABLE I.—COLORIMETER READINGS OF REPLICATE PROCAINE HYDROCHLORIDE SAMPLES[a]

Sample	Reading	Sample	Reading
1	70 5	5	69 5
2	70 5	6	70 0
3	69 5	7	70 0
4	69 5	8	69 5
Mean—69 88		Standard Deviation—0.47	

[a] Each sample contained 1 50 μM procaine hydrochloride

Eight replicates of a procaine hydrochloride standard solution were treated by the method described and the readings obtained on a colorimeter are given in Table I.

A series of procaine hydrochloride-tetracaine hydrochloride mixed solutions was also prepared in which the concentration of procaine was held constant while the tetracaine concentration was varied The solutions were then assayed for their procaine content The purpose of this experiment was to ascertain that the presence of tetracaine hydrochloride had no adverse effect on the procaine hydrochloride determination. As seen in Table II, no interference

TABLE II —ANALYSIS OF KNOWN PROCAINE HYDROCHLORIDE-TETRACAINE HYDROCHLORIDE MIXTURES FOR PROCAINE HYDROCHLORIDE

Sample	Procaine Hydrochloride Actual, μM	Tetracaine Hydrochloride Actual, μM	Procaine Hydrochloride Found, μM	Procaine Hydrochloride, % of Theory
1	1 500	0 000	1 500	100 0
2	1 500	0 060	1 497	99 8
3	1 500	0 150	1 497	99 8
4	1 500	0 300	1 500	100 0
5	1 500	0 600	1 511	100 8
6	1 500	1 500	1 503	100 2
Precision—0.4%				

due to tetracaine was noted—even when the tetracaine hydrochloride was included in over tenfold the concentration present in the commercial mixture.

These data, along with other observations made during the course of this study, indicate that the procedure gives a high degree of precision. Optimum accuracy can be expected in an assay when (*a*) the unknown sample contains from one to two and one-half μM of the local anesthetic being determined, (*b*) standard samples for the preparation of a standard curve are run simultaneously, and (*c*) instrument readings of both unknown and standard samples are taken at the same time interval after addition of the buffer and bleaching reagents Under these conditions the method should be accurate to ± 1–2%. When a previously prepared standard curve is employed, or instrument readings are taken at different time intervals for the unknown and standard samples, then the error will probably be slightly greater. Even under these conditions an accuracy to ± 3–4% can be expected.

SUMMARY

1. The reaction of sodium 1,2-naphthoquinone-4-sulfonate with a series of local anesthetic compounds has been studied.

2. In the case of anesthetics containing a primary amine the reaction quantitatively yielded a highly colored product, the intensity of which was conveniently measured.

3. Secondary and tertiary amines have been shown not to interfere in the assay.

4. Particular application to a commercial anesthetic mixture has been described.

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Catalytic Hydrogenolysis of Halogenated Benzene and Heterocyclic Compounds*

By WILLIAM O. FOYE and LEWIS E. STOYLE, Jr.†

A relatively general procedure for the catalytic hydrogenolysis of aromatically-bound halogen has been found with the use of 10 per cent palladium-on-charcoal. Benzene and pyridine rings remained unaffected during this reaction, but several other heterocyclic rings were reduced or cleaved.

THE REMOVAL of organically-bound halogen is generally anticipated during catalytic hydrogenation of organic compounds, but hydrogenolytic cleavage as a selective method of removing halogen from organic molecules has not been widely investigated. Busch and Stove (1) showed that the halogen in a number of aliphatic and aromatic halides was quantitatively removed by hydrogenation over a palladium-on-calcium carbonate catalyst at atmospheric pressure, but in most cases, the nature of the organic residue was not determined. Using the Adams platinum oxide catalyst, Marvel, *et al.* (2), found that the ring was also hydrogenated under conditions where the halogen was reduced from aromatic halides. More recently, Baltzly and Phillips (3) found that in acid or neutral media, aromatically-bound chlorine could be removed by hydrogenation over a laboratory-prepared palladized charcoal catalyst (4) where the halogen was activated by other substituents. Otherwise, aromatically-bound chlorine was stable under conditions that did not reduce the ring, while bromine similarly bound was generally removed at a moderate rate. Raney nickel has also been used for the quantitative removal of organically-bound halogen (5).

Since a catalyst has been found which is capable of removing chlorine from the s-triazine ring without reduction of the ring (6), it was decided to investigate its use for the removal of other aromatically-bound halogen. The catalyst is 10 per cent palladium-on-charcoal prepared by the American Platinum Works (Newark, N. J.), and it was found, in general, to remove halogen bound to aromatic rings using a pressure of 2–3 atmospheres of hydrogen at room temperature or slightly higher (no attempt was made to hold the temperature constant during the reduction). No attempts to regulate the acidity or basicity

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of the media were made, and no extensive purifications were necessary for the starting materials.

In aromatic halides containing the benzene ring, the ring remained unreduced during removal of chlorine or bromine as expected for palladium catalysts. Although Baltzly and Phillips (3) found that the chlorophenols and chlorobenzoic acids, as well as *p*-bromobenzoic acid, resisted reduction with a palladized charcoal catalyst under conditions where the ring was unreduced, by our procedure both chloro- and bromobenzoic acids and *p*-bromophenol were readily dehalogenated. *p*-Bromobenzoic acid, in fact, was rapidly hydrogenolyzed to benzoic acid with a 78 per cent yield.

Among the heterocyclic halides that were treated by this procedure, only the pyridine ring remained unreduced using 2-chloropyridine. 2-Chloroquinoline was also dehalogenated but partially reduced to 1,2,3,4-tetrahydroquinoline. 2-Chlorobenzoxazole was likewise dehalogenated, but the heterocyclic ring was cleaved leaving aniline hydrochloride as the reduction product. The catalyst was poisoned by sulfur-containing heterocyclic compounds, since addition of fresh catalyst to the inhibited reactions caused further uptake of hydrogen. No identified products were isolated from these reactions. carried out on 2-chlorobenzothiazole and 2-chlorothiophene.

Nitro groups were also readily reduced to amino groups in the presence of halogen, carboxyl, carboxamide, and sulfonamide substituents. Reduction of *p*-bromophenacyl bromide removed only the aromatically-bound bromine to give phenacyl bromide. This result is not surprising, since Baltzly and Phillips (3) found that aliphatically-bound halogen was more resistant to hydrogenolysis than aromatically-bound halogen. With Adams platinum oxide catalyst, both the ring and ketone group of acetophenone are reduced (2). 4,4′-Dibromobiphenyl was the only aromatic halide used that resisted hydrogenolysis by this method.

The compounds hydrogenolyzed with 10 per cent palladium-on-charcoal and their products are recorded in Table I. The times required for removal of halogen are also included, and it can be seen that *para*-substituted electropositive substituents facilitate the removal. This result agrees with the findings of Baltzly and Phillips (3) where amino substituents were shown to be activating. In this connection, it is apparent that nitro groups are reduced to amino groups prior to the removal of halogen, since *p*-chloroaniline and *p*-chloronitrobenzene are reduced in correspondingly similar lengths of time. From a comparison of the times of reaction of the first eight compounds listed in Table I, where the same reaction conditions (molar quantity, concentration, temperature, pressure) prevailed, it may be concluded that in the neutral catalytic hydrogenolysis of aromatic halogen, *para*-substituents affect the ease of halogen removal, and electropositive substituents favor the removal.

EXPERIMENTAL

Materials.—Most of the compounds used were Eastman Organic Chemicals of the best grade available. No purifications were carried out. The remainder were synthesized as follows.

4-Chloro-3-nitrobenzamide.—This compound was prepared from 3-nitro-4-chlorobenzoic acid by means of thionyl chloride and aqueous ammonia. The product was recrystallized from ethanol to give a 75% yield of amide, m. p. 151–153°. Montagne (7) lists a m. p. of 156°.

5-Nitro-2-thiophenecarboxylic Acid.—This compound was prepared by the procedure of Dann (8). The product melted at 155–156°, which agrees with the reported value.

4,4′-Dibromobiphenyl.—This compound was prepared according to the method of Scholl and Neovius (9). An 80% yield of product was obtained after recrystallization from benzene which melted at 164.5–165.5°, which agrees with the reported value.

***p*-Nitrobenzenesulfonamide.**—This was prepared by heating *p*-nitrobenzenesulfonyl chloride for two hours with aqueous ammonia and sodium hydroxide. A 78% yield of the amide was obtained, m. p. 179–180°, which agrees with the reported value (10).

Method.—The apparatus used was a standard Catalytic Hydrogenation Apparatus, Low Pressure, Shaker Type made by the Parr Instrument Co. The catalyst employed was 10% palladium-on-charcoal manufactured by the American Platinum Works, Newark, N. J. The only preparation required before using this catalyst was oven-drying at 60°.

Reductions were carried out using 0.5 Gm. of catalyst per 0.01 mole of organic compound, with a minimum of 0.5 Gm. for any reaction. The pressure of hydrogen used varied from 2–3 atmospheres. The quantity of the substrate varied according to availability and solubility, but was generally from 0.01–0.05 mole. The amount of solvent employed was either 50 or 75 ml., and no purifications were carried out; the best grade available was generally used. The temperature of the reaction was not controlled and was taken as that of the laboratory except in some reductions, particularly where nitro groups were present, where temperature rises were noted. Reduction was considered complete when the hydrogen pressure remained constant for thirty minutes. The time of reduction was taken up to the point where constancy of pressure was first reached, however.

An attempt to maintain constancy of reaction conditions was made for the first eight compounds in Table I. In these cases, 0.01 mole of compound in 50 ml. of solvent with 0.5 Gm. of catalyst was shaken with approximately 2 atmospheres of hydrogen with the starting temperature at approximately 24°.

TABLE I.—HYDROGENOLYSES WITH 10% PALLADIUM-ON-CHARCOAL

Substrate	Moles	Solvent	Temp., °C	Time, min	Product	M. p., °C	Lit. M. p., °C.	Yield, %
p-Chloroaniline	0.01	Methanol	25	3	Aniline·HCl	197–198	198	90
p-Bromoaniline	0.01	Methanol	25	2	Aniline·HBr	150 (s), 275 (m)	286	64
p-Chlorobenzoic acid	0.01	Ethanol	27	5	Benzoic acid	121–122	122	65
p-Bromobenzoic acid	0.01	Abs. ethanol	33	7	Benzoic acid	121–122	122	78
2,4′-Dibromoacetophenone	0.01	Ethyl acetate	25	8	Phenacyl bromide	49–50	50	67
p-Bromophenol	0.01	Methanol	26	2	Phenol			[a]
1-Chloro-4-nitrobenzene	0.01	Methanol	31	5	Aniline·HCl	197–198	198	46
1-Chloro-2,4-dinitrobenzene	0.01	Methanol	31	10	*m*-Phenylene-diamine·HCl	277–278	[b]	89
4-Chloro-3-nitrobenzamide	0.005	Methanol	26	9	*m*-Aminobenz-amide·HCl	238–240	[c]	
p-Nitrobenzenesulfonamide	0.01	Methanol	29	12	Sulfanilamide	165–166	165–166	93
p-Nitrobenzoic acid	0.02	Methanol	28	4	*p*-Aminobenzoic acid	186–187	187–188	89
4,4′-Dibromobiphenyl	0.01	Benzene	26	5	4,4′-Dibromobi-phenyl	163–164	164–165	
2-Chloropyridine	0.05	Methanol	29	30	Pyridine·HCl	80–82	82	80
2-Chloroquinoline	0.025	Methanol	29	135	1,2,3,4-Tetrahydro-quinoline·HCl	179–180	180–181	91
2-Chlorobenzoxazole	0.025	Methanol	30	195	Aniline·HCl	197–198	198	94
2-Chlorobenzothiazole	0.05	Ethyl acetate	27	180	. .			
2-Chlorothiophene	0.05	Methanol	31	180	....			
5-Nitro-2-thiophenecarboxylic acid	0.01	Benzene	29	30			.	

[a] After removal of solvent, a small amount of oily product was isolated which gave a positive phenol test and a negative halogen test.
[b] No literature m. p. could be found for this derivative, so it was converted to the free base, m. p. 61–62°.
[c] No literature m. p. could be found for this derivative, so it was converted to the free base, m. p. 78–79°.

The reduction products were isolated after filtration of the catalyst by evaporation of the solvent. They were then recrystallized to constant melting point. The melting points were taken on a Fisher-Johns block and are uncorrected. Qualitative tests for ionizable halogen were carried out with all the products.

SUMMARY

1. Catalytic hydrogenolysis of aromatically-bound halogen was found to be relatively general using a commercially-prepared 10 per cent palladium-on-charcoal catalyst. Several aryl halides not previously amenable to reduction by this method were readily hydrogenolyzed. The only example noted where the halogen was resistant to reduction was 4,4′-dibromobiphenyl.

2. Nitro groups were also readily reduced to amino groups by this procedure in the presence and absence of halogen removal.

3. Several heterocyclic rings were found to be reduced with this procedure, but the pyridine, as well as the benzene, ring remained unaffected.

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Antipyretic and Toxicity Studies With Acetanilid and *o*-, *m*-, and *p*-Chloroacetanilid*

By M. F. ARGUS, M. P. NEWELL, J. T. HENDERSON, and F. E. RAY

The LD_{50} of *o*-, *m*-, and *p*-chloroacetanilid and of acetanilid was determined in mice and rats. The decreasing order of relative toxicity was the same in both species: *p*-chloroacetanilid, *m*-chloroacetanilid, acetanilid, and *o*-chloroacetanilid. The antipyretic activity of the chloroisomers in the rat decreases in the same sequence as their toxicity.

Ring substitution of the antipyretic drug, acetanild, has an important influence on the metabolic behavior of this compound. Thus, Bray, *et al.* (1), have found that the extent of deacetylation depends on the position of substituents in this molecule. Later, Argus, *et al.* (2), observed that the three ring-substituted monochloroacetanilids display different tissue distribution and urinary excretion patterns following administration to both rats and guinea pigs, suggesting that substitution affects the position and extent of hydroxylation of acetanilid. These alterations in the metabolism of the compound are reflected in changes in the physiological properties of the drug, since with the same dose of *p*-chloroacetanilid and *o*-chloroacetanilid in rats, toxic symptoms were observed only for the former compound.

In the present study a systematic determination of the relative toxicities of *o*-, *m*-, and *p*-chloroacetanilid and unsubstituted acetanilid was made in mice and rats. The effect of the position of ring substitution on antipyretic activity was also investigated.

EXPERIMENTAL

Compounds Used.—The three chloroacetanilids were obtained from the Eastman Chemical Company and were recrystallized from 50% ethanol. Melting points were *o*-chloroacetanilid, 88°: *m*-chloroacetanilid, 72°; and *p*-chloroacetanilid, 177°. Merck acetanilid was recrystallized from water to a melting point of 114°.

Toxicity Experiments.—Female C57bl/6 mice (18 weeks; 20 Gm. ± 2 Gm.) and Sprague-Dawley rats (210-260 Gm.) were employed. Rats were housed 2 to a cage and mice 10 to a cage in an air-conditioned room maintained at 22°. The animals had access to Purina Laboratory Chow and water at all times.

Preliminary tests to determine the dosage range for the toxicity study were made using 2 mice per dose. When the approximate levels were established, groups of 10 mice and 4 rats for each dose level of the isomeric chloroacetanilids and acetanilid were used. Once the LD_{50} values were found for the groups of mice, smaller groups of rats were employed to determine whether the order of toxicity of the compounds was the same in both species. The drugs were administered on a mg./Kg. of body weight basis. Each dose was suspended in 0.6 cc. of 2% Methocel and injected intraperitoneally. The dosages tested are given in Table I. Percentage mortality was based on deaths occurring within seventy-two hours after administration of the drug.

The relative acute toxicities of acetanilid and *o*-, *m*-, and *p*-chloroacetanilid were determined statistically in terms of LD_{50} after Burn, *et al.* (3). In this method the percentage of mortality is plotted against the dosage administered on logarithmic probit paper. The dosage level, at the point where the curve crosses probit 5 on the graph, is taken as the LD_{50}.

Antipyretic Experiments.—Female Sprague-Dawley rats (130–250 Gm.) were employed in groups of 12 animals. Rats were housed two to a cage and all experiments were carried out in a room maintained at 22°. The drugs were given by stomach tube at a concentration of 0.125% suspended in 2% gum acacia. The dosage was 12.5 mg./Kg. body weight. Sixteen hours prior to administration of each compound, fever was induced in the rats by the injection of 15% brewers' yeast (Fisher No. Y-1) suspended in 35% acacia. A subcutaneous injection of 1 cc./100 Gm. body weight produced a 1.5 to 2° rise in the rectal temperature at twelve hours, and this fever then persisted for about eighteen hours. Food (Purina Laboratory Chow) was removed from the animal cages four hours prior to injection of the yeast and twenty hours before administration of the drugs. The animals had free access to water at all times. Groups of eight fevered controls and six nonfevered controls were run simultaneously with each drug-test group.

Rectal temperature determinations were made using a Thermistor Probe (Sargent plain, low range, thermometric element No. S-81620). This probe was connected through a Thermistor Bridge to a Brown Potentiometer and Recorder [Model No. Y153X12V-X-30AKN4(V)]. By inserting the probe in tap water at 34° and 42°, the potentiometer and recorder were standardized, through adjusting the controls on the Thermistor Bridge, so that a reading of "0" on the instrument scale represented 34° and "28" on the instrument represented 42°. A graph paper scale was then superimposed on the instrument scale so that readings to 0.1° could be made. The entire tip of the probe was inserted into the rectum of the rat; five seconds was sufficient for accurate temperature readings.

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RESULTS AND DISCUSSION

Toxicity Experiments.—Table I has been included to give the complete range of toxicities in C57bl/6 mice and in Sprague-Dawley rats, including the doses corresponding to 0 and 100% mortalities These last two values, however, are not used (3) in the probit coordinate system (Figs 1 and 2).

The LD_{50} values obtained for C57bl/6 mice with the probit coordinate system (Fig. 1) are given in Table II as mg /Kg. and mM/Kg. The LD_{50} of *p*-chloroacetanilid is much lower than that of *m*- and

TABLE I.—TOXICITY OF *o*-, *m*-, AND *p*-CHLOROACETANILID AND ACETANILID FOLLOWING INTRAPERITONEAL INJECTION TO C57bl/6 MICE AND SPRAGUE-DAWLEY RATS

Compound	C57bl/6 Mice Dosage,[a] mg /Kg	Mortality, %	Sprague-Dawley Rats Dosage,[b] mg /Kg	Mortality, %
p-Chloro-	182	0	237	25
acetanilid	202	10	246	50
	220	20	255	75
	228	70	278[c]	100
	284	100	284[c]	100
m-Chloro-	500	10	278	0
acetanilid	589	50	302	50
	700	70	360	100
	980	100	425	75
	..	.	500[c]	100
o-Chloro-	700	0	700	25
acetanilid	830	40	760	75
	980	50	830	50
	1370	90	980	75
	. .	.	1370	100
Acetanilid	500	10	425	0
	700	30	500	25
	760	50	589	75
	830	90	700	100
	980	90	.	

[a] Ten animals injected at each dosage level
[b] Four animals injected at each dosage level except as noted
[c] Two animals injected

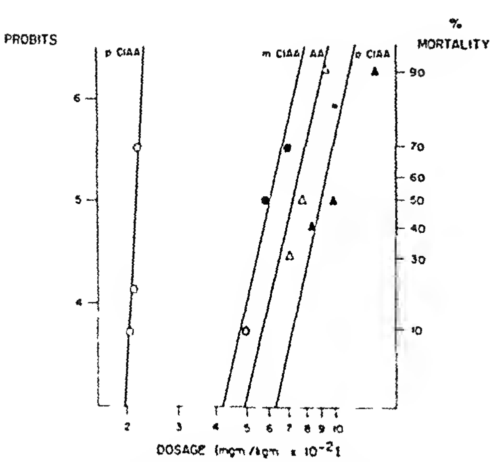

Fig 1.—Dosage of *p*-chloroacetanilid (*p*-ClAA), *m*-chloroacetanilid (*m*-ClAA), acetanilid (AA), and *o*-chloroacetanilid (*o*-ClAA), plotted against percentage mortality in probit units The drugs were administered intraperitoneally to C57bl/6 mice.

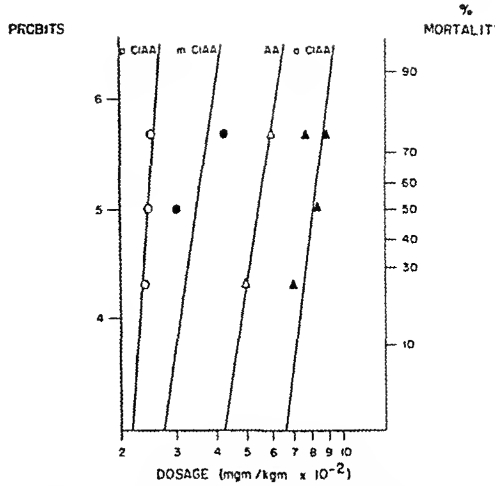

Fig. 2.—Dosage of *p*-chloroacetanilid (*p*-ClAA), *m*-chloroacetanilid (*m*-ClAA), acetanilid (AA), and *o*-chloroacetanilid (*o*-ClAA) plotted against percentage mortality in probit units The drugs were administered intraperitoneally to Sprague-Dawley rats.

o-chloroacetanilid or acetanilid On a mg /Kg. basis, the LD_{50} of *o*-chloroacetanilid is greater than that of acetanilid Yet, on a mM/Kg basis, the LD_{50} of the two drugs is the same. These figures indicate that the introduction of the chlorosubstituent, *per se*, does not increase the toxicity of the compound. It is the position of the substituent which seems to be of importance.

For Sprague-Dawley rats the LD_{50} values (Fig 2 and Table II) show that the relative toxicities of the isomeric chloroacetanilids and acetanilid are the same for the rat as for the mouse *p*-Chloroacetanilid is the most toxic drug, followed in order by *m*-chloroacetanilid, acetanilid, and *o*-chloroacetanilid In rats *o*-chloroacetanilid is less toxic than acetanilid whether the LD_{50} is expressed on a mg /Kg or a mM/Kg. basis

In both species *p*-chloroacetanilid has the narrowest dosage range between 0 and 100% mortality This compound appears to be more toxic to the mice used in these experiments than to the rats In a previous study on the toxicity of orally administered acetanilid in young albino rats, Smith and Hambourger reported an LD_{50} of 800 mg /Kg (4) This is somewhat higher than our value for C57bl/6 mice (715 mg /Kg.) and considerably higher than our findings for Sprague-Dawley rats (540 mg /Kg). In the present investigation, however, acetanilid was administered by the intraperitoneal route, and the differences found for the rat in these two studies are probably due to the difference in the rate of absorption from the gastrointestinal tract and from the peritoneal cavity

In our studies on the excretion pattern of *o*-, *m*-, and *p*-chloroacetanilid we found (2) that the time required for peak excretion of the *p*-compound is four times that needed for the *o*- and *m*-derivatives in the rat It is well known that in rodents, acetanilid is detoxicated mainly by conversion to the more soluble *p* hydroxyaniline (5) Thus, it is not surprising that *p*-chloroacetanilid, in which the *p*-position is blocked, reaches a peak excretion more

TABLE II.—LD_{50} OF *o*-, *m*-, AND *p*-CHLOROACETANILID AND ACETANILID IN C57bl/6 MICE AND SPRAGUE-DAWLEY RATS FOLLOWING INTRAPERITONEAL ADMINISTRATION

Compound	LD_{50} in C57bl/6 Mice[a] mg./Kg.	mM/Kg.	LD_{50} in Sprague-Dawley Rats[a] mg./Kg.	mM/Kg.
p-Chloroacetanilid	225 ± 4.35	1.3 ± .025	245 ± 5.07	1.4 ± .029
m-Chloroacetanilid	610 ± 26.77	3.6 ± .158	350 ± 55.18	2.1 ± .331
Acetanilid	715 ± 31.59	5.3 ± .234	540 ± 28.84	4.0 ± .214
o-Chloroacetanilid	900 ± 74.42	5.3 ± .438	810 ± 53.57	4.7 ± .311

[a] Including standard error.

slowly than the *o*- and *m*-isomers. This longer retention of the *p*-substituted compound by the animal body is now reflected in the greater toxicity of this compound when compared to *o*- and *m*-chloroacetanilid.

Antipyretic Studies.—In the antipyretic experiments with acetanilid, the first depression in the rectal temperature of Sprague-Dawley rats is noted one-half hour after oral administration of the drug in a dosage of 12.5 mg./Kg. (Fig. 3). The greatest depression occurs at three hours with a lowering of 1.1°. The effect begins to diminish at five hours, and at nine hours the depression is only 0 2°. Previously, Smith and Hambourger observed a maximum fall in temperature of only 0 6° when the same dose of acetanilid was given to albino rats (4). In their experiments this maximum temperature decrease occurred between sixty and ninety minutes after administration.

The results obtained with the three isomeric chloroacetanilids are given in Fig. 4. *p*-Chloroacetanilid also brings about a lowering of temperature, beginning 0.5 hour after administration. The greatest depression occurs between one and two and one-half hours and is approximately 0.9°, slightly less than the depression for acetanilid in our experiments. At seven hours the antipyretic effect ceases for the *p*-chloro-derivative. The maximum depression caused by *m*-chloroacetanilid is also 0.9°, which occurs at two and one-half hours after administration. As for the *p*-isomer, no effect is apparent after seven hours. *o*-Chloroacetanilid is the least effective of the four compounds tested. No substantial reduction in temperature is noted until two and one-half hours, and it is only 0.6°. Again no depression of temperature exists after seven hours.

In conclusion, all three isomeric chloroacetanilids have a lower antipyretic activity than acetanilid. In the group of the chloro-isomers the antipyretic activity decreases in the same order as toxicity. There does not seem to be, however, a general correlation between these two pharmacological properties for all four compounds, since *p*- and *m*-chloroacetanilid are more toxic than acetanilid.

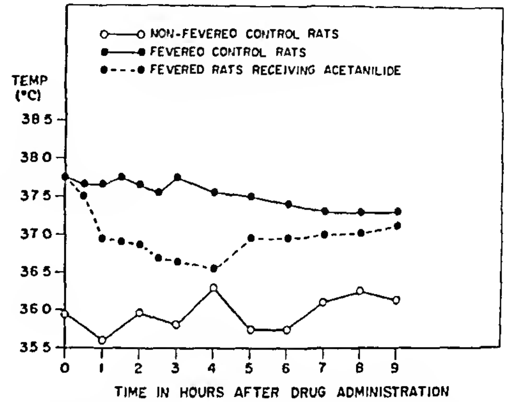

Fig. 3.—Each point on the broken line curve represents the average rectal temperature of 12 Sprague-Dawley rats receiving 12.5 mg. acetanilid/Kg. body weight. The average values for eight fevered control rats and six nonfevered control rats are also plotted.

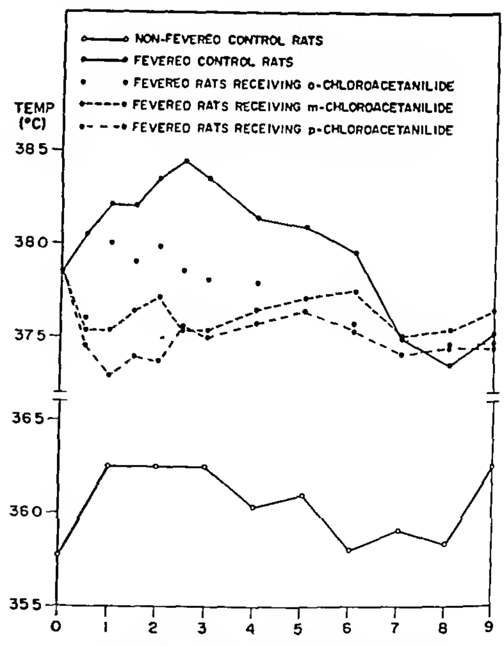

Fig. 4.—Each point on the drug-test graphs represents the average rectal temperature of 12 Sprague-Dawley rats receiving 12.5 mg. *p*-, *m*-, or *o*-chloroacetanilid/Kg. body weight. The average values for eight fevered control rats and six nonfevered control rats are also plotted.

SUMMARY

1. The toxicity expressed as LD_{50}, and the antipyretic activity of acetanilid and of *o*-, *m*-, and *p*-chloroacetanilid were determined in C57bl/6 mice and Sprague-Dawley rats.

2. The relative toxicities on a mg./Kg. basis decrease in both species in the following order: *p*-chloroacetanilid, *m*-chloroacetanilid, acetanilid, and *o*-chloroacetanilid. It has been suggested in

connection with previous studies (2) that the high toxicity of the *p* chloro-isomer may be due to the blocking of *p*-hydroxylation

3. The order of the antipyretic activity in rats of these four compounds is the following acetanilid > *p* chloroacetanilid > *m*-chloroacetanilid > *o*-chloroacetanilid

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The Optical Crystallographic Properties of the Xanthyl Derivatives of Some Barbiturates*

By EDWARD A. JULIAN† and ELMER M. PLEIN

The optical crystallographic properties of the xanthyl derivatives of fourteen commonly-used barbiturates and the micro-melting points of these same derivatives and five additional ones are presented. The crystallographic properties, especially the three principal indexes of refraction, readily serve to identify the compounds in question. The crystallographic properties are of particular value when the melting points of the derivatives are close in range.

One of the physical methods suggested for the qualitative analysis of the barbiturates is optical crystallography employing the petrographic microscope The barbiturates, *per se* (1), and benzyl and phenacyl derivatives of barbiturates (2-5) have been studied by this method

Xanthydrol has been used as a reagent for the identification of barbiturates with melting points as the criteria (6-10). This study presents optical crystallographic data of xanthyl derivatives of barbiturates which can be used as a means of identification.

EXPERIMENTAL

The method followed to obtain xanthyl derivatives of the barbiturates was that of McCutcheon and Plein (10) One gram of xanthydrol (Eastman Kodak Company) was dissolved in 10 ml of glacial acetic acid to which was then added 0 6 Gm of barbiturate The mixture was heated on a water bath for thirty minutes With some derivatives the compound formed immediately, whereas others did not appear until the next day The product was washed free of acid and xanthydrol with 20 ml of ethanol and then dissolved with the heat of a water bath in a minimum quantity of *n*-amyl acetate and acetone (1 1) The solution was filtered into an Erlenmeyer flask and crystallization allowed to proceed slowly In the majority of cases crystals separated out within a few hours

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The compounds were air-dried and checked for purity by using melting points listed in the literature as criteria (except for Butisol, see Table I) (6-10) Melting points were obtained with the Kofler micro-melting-point hot stage.

The critical optical crystallographic properties were determined by methods described by Chamot and Mason (11) and Winchell (12)

Diagnostic properties of crystals in their most frequently occurring orientations were determined by procedures previously described (13)

RESULTS AND DISCUSSION

In Table I are listed the micro-melting points for the various xanthyl derivatives of barbiturates identified by trade names, manufacturers', and generic or chemical names The reagent, xanthydrol, and xanthone, an oxidation product, are included in the table also

The optical properties of xanthyl derivatives of some barbiturates, xanthydrol, and xanthone are listed in Table II The highest index of refraction in the authors' set of liquids was 1 785, hence no value above this figure is reported It was necessary to use crushed specimens in order to obtain some refractive indexes

Many of the xanthyl derivatives are so flattened that they tend to assume a common orientation on the microscope slide In Table III are recorded some optical and crystallographic properties observed on the most frequently occurring orientation of the crystals The optical orientation designated as acute, obtuse, or optic normal indicates that a centered interference figure is found when the crystal, in its most frequently occurring orientation is examined under conoscopic vision The descriptive term, "inclined," indicates that the interference figure is not centered It follows that with an inclined orientation one or both of the refractive indexes for the front view of the crystal cannot be determined within narrow limits and are therefore listed as vari-

TABLE I —MICRO-MELTING POINTS OF XANTHYL DERIVATIVES

Trade Name	Manufacturer[a]	Generic or Chemical Name	Micro-Melting Point[b] of Xanthyl Derivatives, °C
Alurate	Roche Labs Div , Hoffmann-La Roche, Inc.	Aprobarbital	230–231
Amytal	Eli Lilly & Co	Amobarbital	253–254
Butisol[c]	McNeil Labs Inc.	Butabarbital	229–230
Cyclopal	The Upjohn Co.	Cyclopentenylallylbarbituric Acid	235
Delvinal	Merck, Sharp & Dohme	Vinbarbital	227–228
Dial	Ciba Pharmaceutical Products, Inc	Diallylbarbituric Acid	246
Ipral	E R Squibb & Sons	Probarbital	212–214
Luminal	Winthrop Labs , Inc	Phenobarbital	220–221
Nembutal	Abbott Labs	Pentobarbital	223
Neonal	Abbott Labs	Butethal	253–254
Nostal	Ames Co , Inc	Propallylonal	255–256[d]
Ortal	Parke, Davis & Co.	Hexethal	208
Pentothal	Abbott Labs	Thiopental	167–168
Pernoston	Ames Co , Inc	Butallylonal	251[d]
Phanodorn	Winthrop Labs , Inc	Cyclobarbital	258[d]
Sandoptal	Sandoz Pharmaceuticals, Div Sandoz, Inc	Allylbarbituric Acid	248
Seconal	Eli Lilly & Co	Secobarbital	183
Sigmodal	Ames Co , Inc	Amylbromallylbarbituric Acid	199–200
Veronal	Winthrop Labs , Inc	Barbital	246–248
	Eastman Kodak Co.	Xanthone	176–177
	Eastman Kodak Co.	Xanthydrol	122–123

[a] The authors wish to express their appreciation and thanks to the manufacturers who supplied the barbiturates used in this study
[b] The "Kofler" micro melting point hot stage (30–350°) (A H Thomas & Co , Philadelphia, Pa) was used
[c] Butisol (xanthyl derivative) Calculated for $C_{21}H_{22}O_4N_2$ N, 4 89% Found N, 4 73% by the micro-Dumas method.
[d] Decomposed

TABLE II —OPTICAL PROPERTIES OF XANTHYL DERIVATIVES OF SOME BARBITURATES

Xanthyl Derivative	Crystal System[a]	Extinction Angle, Degrees	Optic Sign	Refractive Indexes Alpha	Beta	Gamma	Elongation	Dispersion
Alurate	M	13	+	1 570	1 645	1 725	+	$v > \rho$
Amytal	O	0	−	1 520	1 664	1 730	+	$v > \rho$
Butisol	M	36	−	1 565	1 650	1 716	+	$v > \rho$
Cyclopal	M	36	+	1 580	1 655	1 740	+	$\rho > v$
Dial	M	12	+	1 555	1 645	1 740	+	$\rho > v$
Ipral	T	43	+	1 593	1 617	1 700	±	$v > \rho$
Luminal	M	32	−	1 650	1 696	1 706	±	Crossed
Neonal	M	33	−	1 560	1 645	1 720	−	$v > \rho$
Nostal	M	15	−	1 595	1 685	1 740	+	$v > \rho$
Pentothal	M	21	−	1 608	1 690	1.700	±	$v > \rho$
Pernoston	M	15	−	1 592	1 680	1 730	+	$v > \rho$
Sandoptal	M	8	−	1 565	1 650	1 730	+	$v > \rho$
Sigmodal	M	38	−	1 555	1 675	1 728	+	$v > \rho$
Veronal	M	21	+	1 594	1 620	1 705	+	$v > \rho$
Xanthone	M	28	−	1 680	1 745	1 785	−	$\rho > v$
Xanthydrol	M	27	+	1 590	1 677	>1 785	±	$\rho > v$

[a] M—Monoclinic, D—Orthorhombic, T—Triclinic

able. Extinction angles are measured from the long axis on the front face of each crystal

Figures 1 and 2 are orthographic projection drawings of the crystals showing front, side, and top views These drawings supplement the data presented in Tables II and III and facilitate identification of the barbiturates To determine the appearance of the crystals for side and top views the crystals were observed and rolled in Canada balsam The front view of the crystals is generally the usual orientation Xanthyl derivatives of Amytal and Neonal, however, have ridges down the center of the crystals which make it unlikely that they will be oriented in the position shown. However, this position is presented to illustrate the location of the crystal axes Dashed lines indicate the vibration directions and refractive indexes are recorded for crystals which show consistent values in these directions An asterisk indicates the higher value on views where consistent refractive indexes could not be obtained. Crystal angles measured microscopically are shown in the corners of the diagrams.

Additional descriptions of the reagent and derivatives will facilitate identification of the compounds

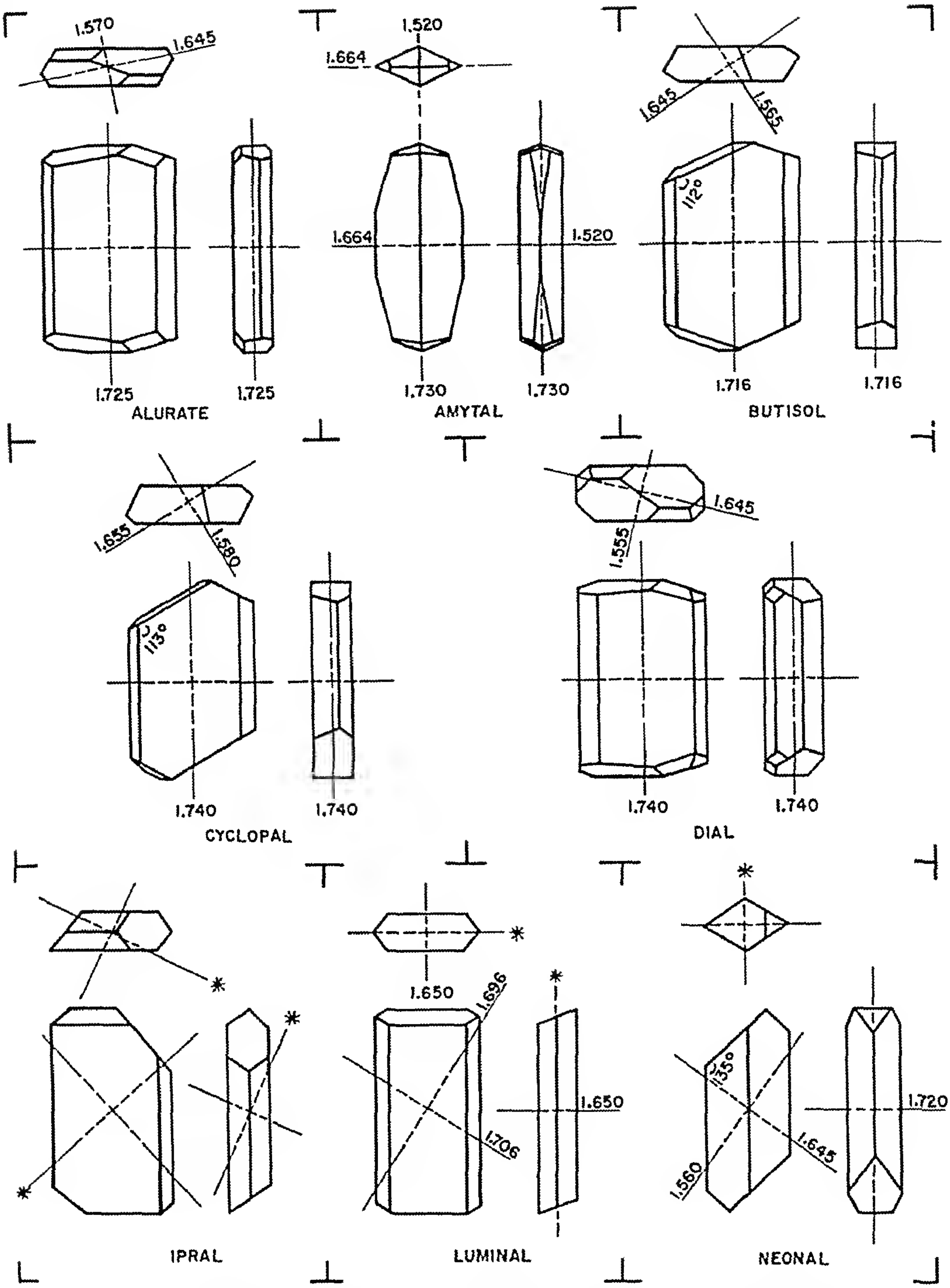

Fig. 1.—Orthographic projections of typical crystals of xanthyl derivatives of barbiturates.

by optical crystallographic methods. The reagent, xanthydrol, is twinned and the variable index across the front face in the usual orientation will be either 1.677 or greater than 1.785. Very few top views of the crystal were found because the crystals are very long and become broken in sampling.

Since xanthydrol is reported to decompose to form xanthone (14), the latter compound was also studied for its optical crystallographic properties. In the course of this investigation, however, no detectable decomposition of xanthydrol occurred. Xanthone formed very long, acicular and taper-

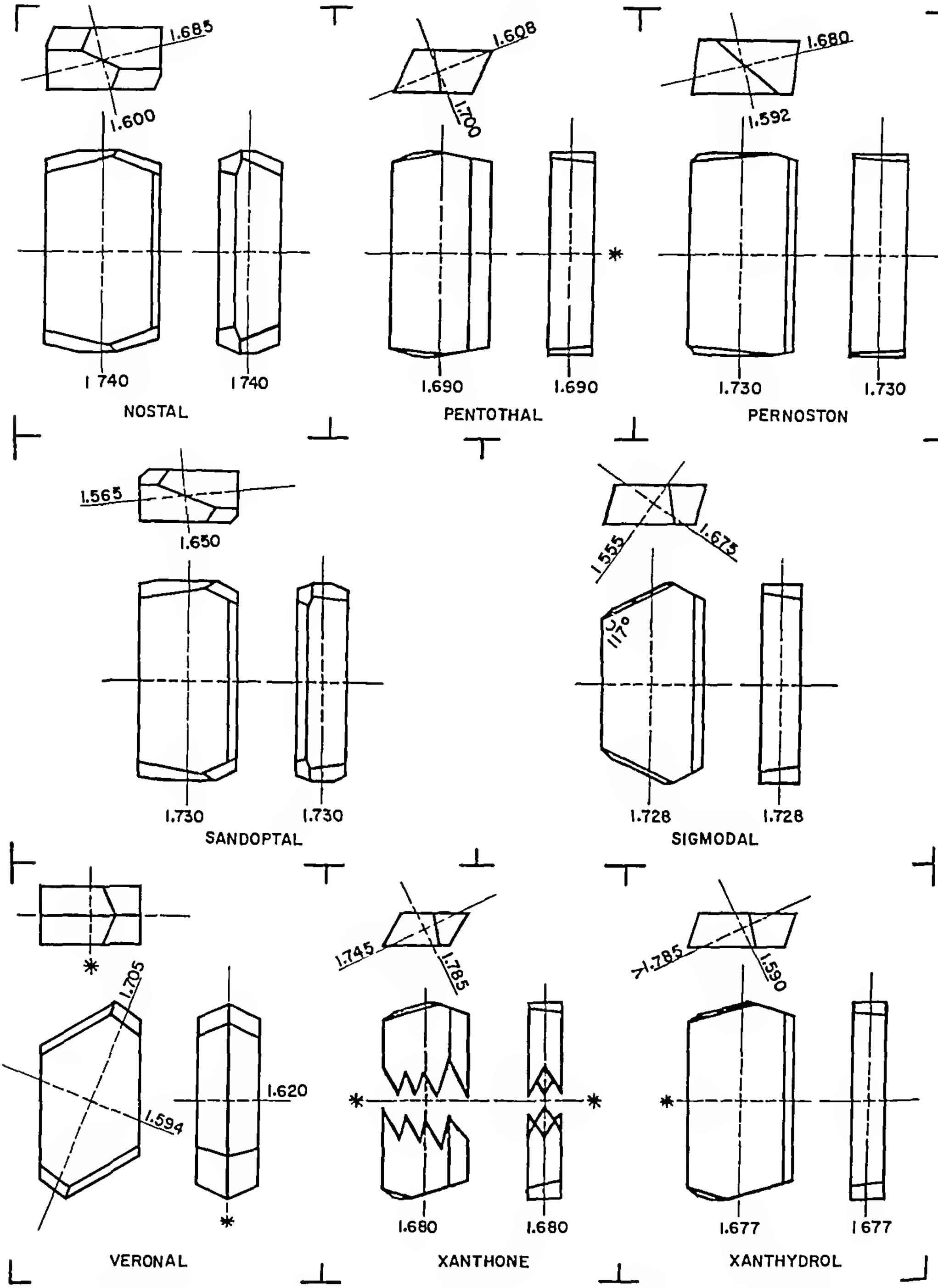

Fig. 2—Orthographic projections of typical crystals of xanthyl derivatives of barbiturates

ing fibrous crystals, twinned along the long axis, and showing very few top views The variable index across the crystal in its usual orientation varied between 1 757 and 1 774 Xanthydrol has, in many respects, the same general appearance as xanthone, however, xanthydrol formed flatter and shorter crystals than xanthone and the optical properties distinguished the two compounds

Xanthyl derivatives of Aluratc, Dial, Nostal, Pernoston, Sandoptal and Veronal have similar prismatic habits, but are easily differentiated by other crystallographic properties

TABLE III.—APPARENT PROPERTIES OF XANTHYL DERIVATIVES OF SOME BARBITURATES FROM MOST FREQUENTLY OBSERVED ORIENTATION

Xanthyl Derivative	Crystal Habit	Optical Orientation	Extinction Angle, Degrees	Refractive Indexes	
Alurate	Prismatic	Inclined Obtuse	0	Variable	1 725
Amytal	Acicular	Acute	0	1 664	1 730
Butisol	Tabular	Inclined Acute	0	Variable	1 716
Cyclopal	Prismatic	Inclined Obtuse	0	Variable	1 740
Dial	Prismatic	Inclined Obtuse	0	Variable	1 740
Ipral	Tabular	Inclined Obtuse	43	Variable	Variable
Luminal	Acicular	Acute	32	1.696	1 706
Neonal	Tabular	Obtuse	33	1.560	1 645
Nostal	Prismatic	Inclined Acute	0	Variable	1 740
Pentothal	Tabular	Inclined Obtuse	0	Variable	1 690
Pernoston	Prismatic	Inclined Acute	0	Variable	1 730
Sandoptal	Prismatic	Inclined Optic Normal	0	Variable	1.730
Sigmodal	Tabular	Inclined Acute	0	Variable	1 728
Veronal	Prismatic	Optic Normal	21	1.594	1 705
Xanthone	Acicular	Inclined Obtuse	0	1.680	Variable
Xanthydrol	Tabular	Inclined Optic Axis	0	1.677	Variable

The xanthyl derivative of Luminal presented a columnar or acicular appearance with twinned cleavage lines parallel to the *c* crystallographic axis. Consequently, most of these twinned crystals did not become extinct under crossed nicols. Single crystals were difficult to find, and these showed crossed dispersion.

The xanthyl derivative of Pentothal crystallized out in yellow, lath-shaped tablets which showed no pleochroism.

Some refractive indexes are reported as variable which is due to the reason explained above. The following are approximate values of some of the intermediate indexes taken from the crystals in their most frequently occurring orientations: xanthydrol, >1 785; xanthone, 1.757; xanthyl derivatives of, Alurate, 1.640; Butisol, 1.625; Cyclopal, 1.645; Dial, 1 630; Ipral, 1.617 and 1.679; Nostal, 1.680; Pentothal, 1 620; Pernoston, 1 675; Sandoptal, 1.570; and Sigmodal, 1.600.

Derivatives which did not form crystals suitable for study were: Delvinal, Nembutal, Ortal, Phanodorn, and Seconal.

The following barbiturates did not form xanthyl derivatives: Evipal (hexobarbital) and Mebaral (mephobarbital), Winthrop Laboratories, Inc.: Mosidal (methallatal) and Gemonil (metharbital), Abbott Laboratories; and Surital (thiamylal), Parke, Davis and Company.

SUMMARY AND CONCLUSIONS

A procedure for the preparation of xanthyl derivatives of barbiturates suitable for crystallographic study has been presented. The optical crystallographic properties of fourteen xanthyl derivatives of barbiturates and the micromelting points of these same derivatives and five additional ones are presented. The optical crystallographic properties of the xanthyl derivatives serve well as a means of identifying the fourteen barbiturates.

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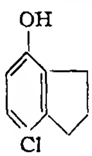
The Biological Properties of Chlorindanol, A New Antiseptic Agent*

By JOHN HAYS BAILEY, FREDERICK COULSTON, and D. A. BERBERIAN

7-Chloro-4-indanol in concentrations of 1:1,000–1:3,000 is rapidly lethal to vegetative bacteria, *Trichophyton sp.*, *C. albicans*, *E. histolytica* cysts and trophozoites, *T. vaginalis*, and spermatozoa *in vitro*. Systemic toxicity is low, it is well tolerated by skin, eyes, genital mucosa, it is nonallergenic, it appears to be suitable for use as topical antiseptic or sanitizer.

CHLORINDANOL, i. e., 7-chloro-4-indanol, was synthesized by Royal A. Cutler (1) of the Chemistry Division of the Sterling-Winthrop Research Institute. The structural formula is



Chlorindanol is a crystalline substance with a molecular weight of 168.6 and m. p. 87.0–89.0° (corr.). It has limited solubility in water but is soluble in dilute alkali and in 95% alcohol to 5% w/v. The antimicrobial activity, toxicity, and irritation thresholds have been studied and are summarized below.

EXPERIMENTAL

The antimicrobial activity of chlorindanol was determined by inoculating representative species of bacteria and fungi into liquid culture media containing varied concentrations of the compound. The lowest concentration of compound which suppressed visible growth, when incubated (for eighteeen hours in the case of most bacteria or for ten days in the case of fungi, yeast-like organisms, and tubercle bacilli), was designated as the 'static concentration. Cultures which showed 'stasis were subcultured into twice the original volume of fresh medium and incubated for an additional forty-eight hours or fourteen days. The minimum drug concentration which prevented growth when subcultured was designated the 'cidal concentration.

Antibacterial Tests.—To a series of tubes containing respectively 3.0, 3.25, 3.5, and 3.75 ml. of culture medium was added, respectively, 1.0, 0.75, 0.5, and 0.25 ml. of a 5 mg./ml. solution of chlorindanol. Dilutions of 1:10 and 1:100 of the drug solution were added in a like manner to replicate series of tubes containing media. Each tube was then inoculated with 1.0 ml. of 1:1,000 dilution in broth of an eighteen to twenty-two hour broth culture of the test organism, resulting in a series of tubes containing concentrations of chlorindanol ranging from 1.0 to 0.0025 mg./ml. contained in a final volume of 5 ml. per tube. End points were determined more exactly by repeating the test with drug concentrations varied at closer intervals within an appropriate range. The procedure was modified in the case of *Clostridium perfringens* by doubling all volumes to result in a final volume of 10 ml. per tube and the culture medium used was Bacto A. C. medium. In the case of *Mycobacterium tuberculosis* the medium used was Youmans' liquid medium and the inoculum consisted of a suspension of tubercle bacilli harvested from Petragnani's medium and adjusted to provide 0.1 mg. per ml. For all other bacteria, tryptose phosphate broth (Bacto) was used, with addition of 2% normal horse serum for growth of streptococci or pneumococci.

Antifungal Tests.—These were performed in the manner described above except that the inoculum consisted of spores prepared by the method of the A. O. A. C. (2) and maltose peptone broth was used as the medium, incubation was at 25° for ten days. In the case of *Candida albicans* the inoculum consisted of a 1:1,000 dilution of a seventy-two hour culture in maltose peptone broth.

Germicidal Efficiency of Chlorindanol.—This was determined as the minimum period of exposure at room temperature required to kill the test organism exposed to given concentrations of germicide. In this procedure 0.5 ml. of an eighteen to twenty-two hour broth culture was added to 5 ml. of an aqueous solution of chlorindanol and rapidly mixed. At the end of various periods of exposure, 0.05 ml. of the test mixture was removed to 10 ml. of F. D. A. broth containing 0.7 mg. of lecithin per ml. of medium as an inactivator, and incubated for forty-eight hours at 37°. Absence of visible growth was taken to mean effective germicidal action within the exposure time represented by the sample.

The results of tests for antibacterial activity are shown in Table I. Chlorindanol was bacteriostatic and bactericidal for the 20 species of Gram-negative bacteria and the 10 species of Gram-positive bacteria. In general, chlorindanol was somewhat more active against Gram-negative than against Gram-positive bacteria. It is noteworthy that the difference between bacteriostatic concentration and bactericidal concentration was always small and in a number of instances the values were identical, suggesting that the action of the compound was rapid even at limiting dilutions. Chlorindanol was ineffective against spores of *Bacillus subtilis*, but it was effective against a strain of *C. perfringens* which is meagerly sporogenic. In terms of dilution, vegetative forms of all the species and strains tested were susceptible to chlorindanol 1:2,500 (one strain of *Pseudomonas*) to 1:25,000 (*M. tuberculosis*).

The germicidal efficiency of chlorindanol is shown by the data presented in Table II. *Staphylococcus aureus* 209 was killed within seven and one half

* Received August 13, 1958, from the Biology Division, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

TABLE I.—ANTIBACTERIAL ACTION OF CHLORINDANOL FOR GRAM-NEGATIVE AND GRAM-POSITIVE ORGANISMS

	Minimum Concentration Required, mg./ml. 'Stasis	Kill
Gram-negative		
Aerobacter aerogenes	0.2	0.2
Brucella abortus 11192	0.03	0.07
B. melitensis L-1	0.05	0.1
B. suis 1744	0.06	0.1
Desulfovibrio desulfuricans 7757	0.03	0.07
Escherichia coli 198	0.07	0.09
E. coli 4157 G	0.06	0.08
Eberthella typhi Hopkins	0.05	0.07
Klebsella pneumoniae 48	0.1	0.1
Pasteurella boviseptica Harvard	0.05	0.07
Proteus vulgaris 9920	0.1	0.1
P. mirabilis 9921	0.1	0.1
Pseudomonas aeruginosa N.O.	0.2	0.4
P. aeruginosa 211	0.4	0 4
Salmonella enteritidis 588	0.06	0.08
S. schottmuelleri	0.07	0.1
S. paratyphi 9150	0.07	0.1
Shigella dysenteriae Shiga.	0.05	0.07
S. paradysenteriae	0 06	0.07
Vibrio cholerae 23	0.03	0.04
Gram-positive		
Bacillus subtilis	0.05	>1.0
Clostridium perfringens Mich.	0.06	0.1
Diplococcus pneumoniae I	0.03	0.05
D. pneumoniae II	0.03	0.05
Mycobacterium smegmatis	0.04	0.05
M. tuberculosis H37RV	0 02	0.04
Staphylococcus aureus 209	0.06	0.15
S. aureus SWRI	0.06	0.06
Streptococcus faecalis 10Cl	0.06	0.07
S. pyogenes C203	0 09	0.2
S. viridans 9811	0.08	0.15

minutes by a dilution of 1:1,500 at room temperature. *Eberthella typhi* Hopkins was the most susceptible of the organisms tested by this procedure. Relatively resistant strains of *P. aeruginosa* were killed by exposure to 1:1,000 for five minutes.

Table III shows that chlorindanol is effective *in vitro* in low concentrations against *Candida albicans* and a variety of fungi when exposure is prolonged. In a fungicidal efficiency test, it was demonstrated that *C. albicans, T. mentagrophytes*, and *A. niger* were killed by exposure to 1:1,000 and 1:2,000 dilutions for five minutes and sixty minutes, respectively (Table IV).

Conventional phenol coefficient values were determined for chlorindanol by the so-called F. D. A. procedure, using several species of bacteria as the test organisms. These values are summarized in Table V. These values were not significantly reduced when the subcultures were made into lecithin inactivator broth.

Chlorindanol 1:5,000 was amebacidal for *Endamoeba histolytica* when incorporated in Hansen's egg infusion medium, inoculated with the sediment from a pool of rich cultures, and incubated at 36° for thirty hours; a 1:10,000 dilution reduced the number of amebae per drop of sediment from 590 to 9, and 1:20,000 dilution was ineffective. A 1:2,500 dilution killed a heavy inoculum of cysts of *E. histolytica* in thirty minutes but not in ten minutes exposure.

Chlorindanol was tested for trichomonacidal activity by mixing various dilutions in isotonic saline solution with cecal content from hamsters containing approximately 3 million trichomonads (*Trichomonas muris* and *T. minuta*) per ml. Chlorindanol 1:2,000 immobilized all trichomonads in an average time of one minute and six seconds, 1:3,000 killed in three minutes, 1:4,000 killed in twelve minutes and forty-two seconds, and 1:5,000 killed in eighteen minutes and thirty seconds. When chlorindanol was added to rich cultures of *Trichomonas vaginalis* of human origin, in Johnson's medium (3), a dilution of 1:2,000 completely immobilized all trichomonads in less than one minute, 1:4,000 immobilized in two minutes, and 1:8,000 immobilized in thirteen minutes when immobilization time was measured from the moment of mixture of the culture with an equal volume of chlorindanol.

The action of chlorindanol on human spermatozoa was determined on two specimens of semen, from different patients, received in a viable condition from a collaborating physician. In accordance with the procedure of Brown and Gamble (4), a small quantity of semen was drawn into a capillary pipet, followed by an equal volume of drug dilution. The material was thoroughly mixed on a microscope slide and sealed under a cover glass. Final dilutions of 1:2,000, 1:3,000, 1:4,000, 1:5,000, 1:6,000, 1:8,000, and 1:10,000 chlorindanol were tested. The rate of immobilization was determined by continuous observation under the microscope. There was some variation between replicate samples, obviously due to variations in uniformity of mixing. However, it is clear that chlorindanol is a highly effective spermicide. The dilutions of 1:2,000 and 1:3,000 immobilized immediately; 1:4,000 immobilized in an average time of two minutes and thirty seconds, and three minutes; 1:5,000 immobilized in ten minutes and forty-eight seconds and eleven minutes and twelve seconds (range 3-18 and 3-25 minutes); 1:6,000 immobilized in twenty-three minutes and 1:8,000 in fifty-three minutes; 1:10,000 was not completely effective in three hours.

SAFETY EVALUATION OF CHLORINDANOL

The systemic toxicity of chlorindanol and its effect on tissues have been studied under a variety of experimental conditions as summarized below.

Acute toxicity (5).—The toxicity of single graduated doses of chlorindanol was determined by administration either orally, intravenously, or intraperitoneally to groups of ten male Swiss (Webster) albino mice weighing 22 ± 2.0 Gm. For oral and intraperitoneal medication the drug was prepared as a 4% stock solution in dilute NaOH and adjusted to pH 10.0. Appropriate aqueous dilutions were prepared to provide the desired dose levels in a volume of 0.01 ml./Gm. of body weight; oral administration was by stomach tube. For intravenous administration a 1.0% solution of chlorindanol was prepared and injection into the tail vein was made at a constant rate of 1.0 ml. per minute. The animals were observed for symptoms of acute toxicity over a peric of several hours following medication and for delay symptoms over the seven days following medicatio

TABLE II.—GERMICIDAL EFFICIENCY OF CHLORINDANOL AT ROOM TEMPERATURE

Test Organism		Maximum Dilution Killing, Min. ½	1	2½	5	7½	10	15
S. aureus 209	a)	1:1,000	1:2,000	1:2,500	...	...	...	...
	b)		...	..	1:1,000	1:1,500	1:1,500	1:1,500
E. typhi Hopkins		1:1,000	1:3,000	1:3,000	1:4,000	1:4,000	1:4,000	1:4,000
Es. coli (2 strains)		.	..	. .	1:3,000	...	1:3,500	1:4,000
P. aeruginosa 211		..	...	...	1:1,000	...	1:1,500	1:1,500
P. aeruginosa N.O.		...	...	.	1:1,000	...	1:1,500	1:1,500
M. tuberculosis H 37 Rv[a]		..	.	...	1:1,000	...	1:2,500	...

[a] Lethal effect confirmed by guinea pig inoculation.

TABLE III.—ANTIMICROBIAL ACTION OF CHLORINDANOL FOR CERTAIN YEAST-LIKE ORGANISMS AND FUNGI

	Minimum Concentration Needed, mg. ml. 'Stasis	Kill
Aspergillus niger	0.025	0 3
Candida albicans	0 03	0.03
Pityrosporum ovale	0.05	0 05
Trichophyton gypseum	0 025	0 03
T. interdigitale	0 025	0.025
T. mentagrophytes	0 0125	0.025

TABLE IV.—FUNGICIDAL EFFICIENCY

Organism	Min. Required to Kill 1 1,000	1:2,000	1:4,000
C. albicans	<5	<5	30
T. mentagrophytes	<5	<5	<5
A. niger	<5	<5	60

TABLE V.—PHENOL COEFFICIENT VALUES OF CHLORINDANOL

	Average P. C. at 20° Without Serum Av. P. C.	Av. Limiting Diln.	With 10% Serum P. C.	Av. Limiting Diln.
S. aureus 209	27.0	1625	12.2	733
E. typhi Hopkins	46.4	4187	27.7	2,500
Es. coli 198	43.7	3500	25.8	2,333
Es. coli 4157 G	53.0	4166	22.2	2,000
Sal. enteritidis 588	56.2	4500	22.2	2,000
P. aeruginosa 211	21.8	1750	12.9	1,000
P. aeruginosa N.O.	19.3	1550	14 2	1,000

As a measure of toxicity, the LD_{50} was calculated by the method of Miller and Tainter (6) at twenty-four hours and at seven days after medication.

The LD_{50} values obtained are presented in Table VI. The symptoms of acute oral toxicity consisted of slight to severe respiratory depression and general depression, related to the magnitude of the dose. The deaths occurred within two to fifteen hours after medication. Intravenous injection of toxic doses caused hyperexcitability, tonic and clonic convulsions, and death due to respiratory arrest within one to ten minutes after injection; there were no delayed deaths. Intraperitoneal injection of toxic doses

TABLE VI.—ACUTE TOXICITY OF CHLORINDANOL FOR 22-GM. MICE

Route of Administration	$LD_{50} \pm$ S. E., mg./Kg. at 24 hrs.	at 7 days
Intravenous	48 ± 2	48 ± 2
Intraperitoneal	188 ± 30	96 ± 22
Oral[a]	960 ± 52	920 ± 35

[a] Administered as an alkaline solution at pH 10.0. When chlorindanol was administered as a suspension in 1% gum tragacanth for five consecutive days the LD_{50} for mice was greater than 1,585 mg./Kg./day and for rats was calculated to be 1,650 ± 265 mg./Kg./day.

caused severe general depression and the deaths occurred sporadically over a period of several days. The values given in Table VI indicate that chlorindanol is not a highly toxic compound.

Subacute toxicity.—Two types of subacute toxicity tests were carried out. The determination of an LD_{50} on the basis of daily doses administered by stomach tube for five consecutive days, followed by observation for seven subsequent days, is particularly useful for bringing out any tendency for cumulative delayed toxic manifestations. Secondly, a three weeks toxicity test carried out at relatively high dose levels is useful in providing a measure of the degree of tolerance to be expected from chronic intake or absorption of the drug.

Five-day subacute toxicity tests were carried out both in mice and in rats (Sprague-Dawley strain). Chlorindanol was administered to groups of five male albino mice as single daily doses of 795 and 1,585 mg./Kg. for five consecutive days. The drug was administered as a suspension in 1.0% gum tragacanth solution. A control group received only the vehicle for five days. Hematological studies were carried out on all surviving mice one day before they were sacrificed and autopsied. The mice which received 795 mg./Kg. daily developed moderate respiratory and general depression within twenty to thirty minutes after each dose. The depression persisted for four to five hours after the first dose and was of progressively shorter duration thereafter. There were no deaths at this dose level and body weight gain did not differ significantly from that of the controls. All of the mice which received the daily dose of 1,585 mg./Kg. showed severe respiratory and general depression within twenty to thirty minutes after medication; these animals remained prostrate for four to five hours after the first dose but the symptoms became progressively less severe. At the high dose level one mouse died about 12 hours after the first dose but all others survived with only moderate weight loss during the medication period and weight was gained at a normal rate after the last dose. The blood studies did not show any ab-

normal deviation in number or morphology of leucocytes or erythrocytes, and pathological changes attributable to the medication were not found at autopsy.

Chlorindanol was administered orally once daily for five consecutive days to three groups of ten male albino rats at dose levels of 800, 1,600 and 3,200 mg./Kg. The drug was suspended in 1.0% gum tragacanth. A control group of ten rats received only the vehicle. The animals were observed for seven days after the last dose and blood studies were carried out on the control rats and the seven surviving rats of the 1,600 mg./Kg. group on the sixth post-medication day; all survivors were sacrificed and autopsied on the seventh day.

All rats which received 800 mg./Kg. daily for five days were normal in appearance and behavior throughout the experiment. Four of the ten rats in the 1,600 mg./Kg. group developed blood tinged nasal exudate and three of them died (two on the third day and one on the fourth day of medication). All of the rats on the high dose level (3,200 mg./Kg.) developed bloody secretions, diarrhea, and died by the end of the fourth day of medication. The body weight gain of the rats on the low dose was slightly depressed, and the 1,600 mg./Kg. dose caused moderate depression of weight gain during the medication period; all survivors gained at a normal rate as soon as medication was stopped. There was no significant deviation from normal leucocyte or erythrocyte counts, differential count or hemoglobin concentration of the control rats or seven survivors of the 1,600 mg./Kg. group. Significant gross or histopathological changes were not found on autopsy of all surviving rats. The 5-day LD_{50} of chlorindanol in rats was calculated to be 1,650 ± 265 mg./Kg. per day. It is apparent from this study that chlorindanol, orally, is less readily absorbed, and consequently less toxic, when administered as a suspension than when administered as an alkaline solution.

Three-Weeks Subacute Toxicity Test in Rats.—Chlorindanol was administered orally, by stomach tube, to three groups of ten male albino Sprague-Dawley rats in single daily doses of 200, 400, and 800 mg./Kg. A total of 18 doses was administered over a period of twenty-one days. The drug was given as a suspension in 1.0% gum tragacanth. A control group of ten rats received only the vehicle. The animals were weighed three times a week and carefully observed for signs or symptoms of toxicity. Hematological studies were carried out on all of the animals four days before the end of the experiment. At the end of the experiment all animals were sacrificed with ether, the thoracic and abdominal viscera were examined for gross lesions, and portions of each organ were fixed in Zenker-formalin for histological study.

All of the animals remained normal in appearance and behavior throughout the experiment. One rat on the low dose died of intercurrent pneumonia on the tenth day. The average growth rate of the rats on the low dose was similar to that of the control group throughout the experiment. The growth of the rats on the 400 and 800 mg./Kg. dosage was depressed in relation to size of the dose; body weight was 11.3% and 38% less than the control group at the end of the experiment. The blood studies did not show any abnormal deviation in the total erythrocyte or leucocyte count, in hemoglobin concentration, differential count, or hematocrit. Pathological changes in organs or tissues, attributable to medication with chlorindanol, were not found.

Irritation Studies.—Experiments were carried out to determine the capacity of chlorindanol to cause toxic irritation to skin, eyes, and genital mucosa of rabbits. The procedures used were those of Draize, Woodard, and Calvery (7) and the severity of ocular inflammation was scored by the method of Hoppe, *et al.* (8).

Skin irritation tests were carried out on 14 rabbits, of which 12 were treated with chlorindanol and two were treated with control solutions. The area of the clipped back and sides of each animal was marked off into quadrants. The skin of alternate quadrants was abraded by means of two pairs of intersecting scratches; each scratch was one centimeter long. Each test area was covered with a 4 x 4 cm. sterile gauze pad secured to the skin by means of adhesive tape. An equal number of abraded and intact areas of skin were treated with powdered crystalline chlorindanol and with a 2.0% solution of the drug in 46% polyethylene glycol (PEG-400). Controls received only the vehicle. The test materials were introduced as uniformly as possible under the pads. The treatment consisted of 0.5 Gm. of pure chlorindanol powder or 0.5 ml. of the 2.0% solution. After application of the medication, the entire trunk of each rabbit was wrapped in polyethylene sheet and a heavy cloth. The wrappings and the pads were removed after twenty-four hours for examination and scoring; additional observations were made at seventy-two hours and seven days. For evaluation, the scores at twenty-four and seventy-two hours were averaged separately and the derived values were then averaged to give the primary irritation index.

According to Draize, *et al.* (7), "compounds producing combined averages (Primary irritation indexes) of 2 or less are only mildly irritating whereas those with indexes from 2–5 are moderate irritants, and those above 6 are considered severe irritants." In the experiment described above, the combined average for chlorindanol powder was zero, for the 2.0% solution in PEG-400 the index value was 0.05, and for the vehicle control the index was zero. It is concluded that under the conditions of the experiment, chlorindanol is not a primary irritant for the skin of the rabbit.

Eye irritation tests were carried out according to Draize, *et al.* (7), and Hoppe, *et al.* (8). The concentrations of chlorindanol tested were 0.5, 0.75, 1.0, and 1.5% in 67% PEG-400. Twenty-two rabbits were used for the experiment, five for each dose level and two for vehicle controls. One-tenth milliliter of the test solution was instilled into the conjunctival sac of the right eye of each rabbit. The condition of the medicated eye was compared with that of the unmedicated left eye at twenty-four, forty-eight, seventy-two, and ninety-six hours, and at seven days. The results indicated that the 0.5% solution was completely tolerated; three of five animals receiving 0.75% solution showed transitory mild hyperemia which disappeared within twenty-four hours; the two higher doses caused moderate hyperemia which was apparent at twenty-four, forty-eight, and ninety-six hours but not at one hundred-twenty hours; partial clouding of the cornea was observed in two rabbits of the 1.0% group and four of five rabbits of the 1.5% group but all eyes were normal by

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Test Organism		Maximum Dilution Killing, Min 1/2	1	2 1/2	5	7 1/2	10	15
S aureus 209	a)	1 1,000	1 2,000	1 2,500				
	b)				1 1,000	1 1,500	1 1,500	1 1,500
E typhi Hopkins		1 1,000	1 3,000	1 3,000	1 4,000	1 4,000	1 4,000	1 4,000
Es coli (2 strains)					1 3,000		1 3,500	1 4,000
P aeruginosa 211					1 1,000		1 1,500	1 1,500
P aeruginosa N O					1 1,000		1 1,500	1 1,500
M tuberculosis H 37 Rv[a]					1 1,000		1 2,500	

[a] Lethal effect confirmed by guinea pig inoculation

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	Minimum Concentration Needed, mg ml 'Stasis	Kill
Aspergillus niger	0 025	0 3
Candida albicans	0 03	0 03
Pityrosporum ovale	0 05	0 05
Trichophyton gypseum	0 025	0 03
T interdigitale	0 025	0 025
T mentagrophytes	0 0125	0 025

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Organism	Min Required to Kill 1 1 000	1 2 000	1 4 000
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A niger	<5	<5	60

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E typhi Hopkins	46 4	4187	27 7	2,500
Es coli 198	43 7	3500	25 8	2,333
Es coli 4157 G	53 0	4166	22 2	2,000
Sal enteritidis 588	56 2	4500	22 2	2,000
P aeruginosa 211	21 8	1750	12 9	1,000
P aeruginosa N O	19 3	1550	14 2	1,000

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Eye irritation tests were carried out according to Draize, *et al.* (7), and Hoppe, *et al.* (8). The concentrations of chlorindanol tested were 0.5, 0.75, 1.0, and 1.5% in 67% PEG-400. Twenty-two rabbits were used for the experiment, five for each dose level and two for vehicle controls. One-tenth milliliter of the test solution was instilled into the conjunctival sac of the right eye of each rabbit. The condition of the medicated eye was compared with that of the unmedicated left eye at twenty-four, forty-eight, seventy-two, and ninety-six hours, and at seven days. The results indicated that the 0.5% solution was completely tolerated; three of five animals receiving 0.75% solution showed transitory mild hyperemia which disappeared within twenty-four hours; the two higher doses caused moderate hyperemia which was apparent at twenty-four, forty-eight, and ninety-six hours but not at one hundred-twenty hours; partial clouding of the cornea was observed in two rabbits of the 1.0% group and four of five rabbits of the 1.5% group but all eyes were normal by

the seventh day. The control solution of the 67% PEG-400 did not cause any inflammation Under the conditions described, chlorindanol 0 5% was nonirritating, 0.75% was very mildly irritating, 1.0%, and 1.5% were only moderately irritating Chemosis and discharge were minimal at the higher concentration. In an additional experiment on five rabbits, a 2.0% solution of chlorindanol in 46% PEG-400 was rated as only moderately irritating and all eyes were clear at seven days; the maximum average total score, at forty-eight and seventy-two hours, was 10.2 of a maximum possible score of 20.

An experiment was carried out to determine the effect of chlorindanol on the penile mucosa of the rabbit. Three adult male albino rabbits were used to test the irritancy of 2.0% chlorindanol solution in 46% aqueous PEG-400 A fourth rabbit was used to test the vehicle, as a control. The penis and urethral orifice of each rabbit was thoroughly wetted with 0.2 cc. of test solution, which was not removed Observations were made at one hour, twenty-four, and forty-eight hours after medication. There was no visible evidence of inflammation after application of 2.0% chlorindanol in PEG

Allergenicity.—An experiment was carried out in guinea pigs to test the allergenicity of chlorindanol in accordance with the procedure of Draize, *et al.* (7). Seven male guinea pigs, ranging from 325 to 450 Gm in weight, were used. Five guinea pigs were prepared by a series of ten intradermal injections of an 0.1% solution of chlorindanol in 10% PEG-400, and two guinea pigs received similar "sensitizing" injections of 10% PEG-400. The injections were made intradermally, three times a week until a total of ten had been completed. The first injection was a single dose of 0.05 ml followed by nine injections of 0.1 ml. At the time of each injection, the area of any resulting erythema and induration was recorded. Two weeks after the last preparative dose, each guinea pig was challenged for evidence of induced hypersensitivity by an intradermal injection of 0 05 ml. of a fresh 0 1% solution of chlorindanol in PEG-400, and for control purposes each animal also received 0.05 ml. of 10% PEG-400. The sites of injection were observed for immediate reaction, and again twenty-four hours after injection. Areas of erythema were measured for comparison with the response to the first preparative injection. It was not possible to distinguish between the wheals produced by the PEG-400 alone and PEG-400 containing chlorindanol, or between the primary response and the challenge response. It was concluded that chlorindanol is not an allergenic substance under the conditions described

Solutions of 0 5%, 1.0%, and 2 0% chlorindanol in 46% aqueous PEG-400 did not modify the course of healing of incisions 1 cm. in length made into the dermal layer of skin of six adult albino rabbits. Each incision was covered with a 4-cm. square sterile gauze pad and 0.5 ml. of test solution was applied daily for three days. For the first three days the solution was introduced under the pad and the trunk of the animal was securely wrapped. After the third day the wrappings were omitted and pads containing test solution were held in contact with the wound for one minute twice daily for seven days, at which time the experiment was terminated. During the course of observation there was no evidence of secondary infection or of irritation attributable to the solutions applied.

As the result of the experiments described above, it is concluded that the intrinsic toxicity of chlorindanol is low, it is nonallergenic, and it is well tolerated by skin, eyes, or genital mucosa

SUMMARY

Chlorindanol 1:1,000 to 1:3,000 is rapidly lethal to vegetative bacteria, *Trichophyton* sp., *Candida albicans*, *Endamoeba histolytica* cysts and trophozoites, *Trichomonas vaginalis*, and spermatozoa *in vitro*. Oral toxicity is low, but is somewhat greater when administered in alkaline solution (LD_{50} for the mouse = 920 ± 35 mg./Kg.) than when suspended in gum tragacanth (LD_{50} for five consecutive daily doses = >1,500 mg./Kg./day). In a three weeks toxicity test, rats completely tolerated daily oral doses of 200 mg./Kg. Dose levels of 400 and 800 mg./Kg. caused some retardation of weight gain but there were no deaths. Extensive irritation studies showed that chlorindanol solutions of less than two per cent in PEG-400 were well tolerated by skin, conjunctiva, and genital mucosa of rabbits, and were also shown to be nonallergenic for guinea pigs. A two per cent solution in forty-six per cent PEG-400 did not interfere with healing of wounds. The properties of chlorindanol indicate that it is suitable for topical antiseptic or sanitizing purposes.

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The Analysis of Digoxin Preparations*

By ALBERT E. H. HOUK, THOMAS G. ALEXANDER, and DANIEL BANES

Methods are proposed for the analysis of crystalline powders, tablets, injections, and elixirs containing digoxin. The glycoside is assayed by means of an improved *m*-dinitrobenzene reagent, and its purity is determined by chromatographic and colorimetric tests.

An earlier report from this laboratory (1) described a procedure employing *m*-dinitrobenzene in weakly alkaline solution for the colorimetric determination of digoxin. Further studies on the chromogenic reaction showed that small quantities of water in the alcoholic reagent profoundly influenced both the purity and the intensity of the color developed. That reagent produced colors with absorption maxima at 480 and 600–615 mμ. Use of the reagent proposed below yielded more intense colors with a single peak in the visible region at 620 mμ. The colors attained their maximum absorbances in about ten minutes at 25° under the conditions of the test, with only slight deviation from the Beer-Lambert law.

PROPOSED METHODS OF ANALYSIS

Alkaline Dinitrobenzene Reagent

(*a*) Prepare a 5% solution of *m*-dinitrobenzene in benzene, and store in a glass-stoppered brown glass bottle. (*b*) Mix 1 ml. of 10% tetramethylammonium hydroxide solution with 140 ml. of absolute alcohol, titrate a portion with 0.01 *N* hydrochloric acid, and adjust the remainder to 0.008 *N* with absolute alcohol. Just prior to use, mix 60 ml. of (*a*) with 40 ml. of (*b*).

Assay Procedure

Sample Preparation.—(*a*) *Crystalline Digoxin.*—Prepare an alcoholic solution containing 125 μg. of digoxin per ml. Transfer 10.0 ml. to a separator, add 50 ml. of water and 1 ml. of 2 *N* sulfuric acid, and extract with three 30-ml. portions of chloroform. Wash each chloroform extract in a second separator by shaking with 10 ml. of water and 1 Gm. of ground anion-cation exchange resin,[1] and filter through a pledget of cotton moistened with chloroform into a 100-ml. volumetric flask. Dilute to the mark with chloroform, and mix well. This solution is the *Assay Preparation.*

(*b*) *Elixirs and Injections.*—Transfer an aliquot containing 1.25 mg. of digoxin to a separator, and proceed as directed under *Crystalline Digoxin* beginning with the words: "Add 50 ml. of water and 1 ml. of 2 *N* sulfuric acid . . . ."

(*c*) *Tablets.*—Weigh into a 100-ml. beaker an accurately measured portion of powdered tablets equivalent to 1.25 mg. digoxin. Add 10 ml. of boiling alcohol, and stir. Cover with a watch glass, and allow to stand for twenty minutes at 60° with frequent stirring. Cool, wash into a separator quantitatively with 30 ml. of chloroform and 50 ml. of water, add 1 ml. of 2 *N* sulfuric acid, and proceed as directed under *Crystalline Digoxin* beginning with the words: "extract with three 30-ml. portions of chloroform . . . ."

Standard Preparation.—An alcoholic solution containing 25.0 μg. of U. S. P. Reference Standard Digoxin per ml.

Colorimetric Determination.—Pipet 5.0 ml. of the *Standard Preparation* and 10.0 ml. of the *Assay Preparation* into similar Erlenmeyer flasks, and evaporate to dryness on the steam bath with the aid of a current of air. Cool and add to each dry residue 5.0 ml. of freshly prepared alkaline dinitrobenzene reagent. Let stand five minutes at a temperature not exceeding 30°, with frequent mixing. Determine the absorbances of the developing blue colors relative to the reagent blank at 620 mμ at one-minute intervals, using matched 1-cm. cells and a suitable spectrophotometer. Record the maximum absorbance of the aliquot from the *Assay Preparation* as *A* and that from the *Standard Preparation* as *S*. The quantity, in mg., of $C_{41}H_{64}O_{14}$ in the *Assay Preparation* is given by the expression 1.25 *A*/*S*.

Other Digitoxosides.—Pipet 20.0 ml. of the *Assay Preparation* and 10.0 ml. of the *Standard Preparation* into separate 50-ml. Erlenmeyer flasks and evaporate to dryness on the steam bath with the aid of a current of air. Cool, add 4.0 ml. of Keller-Kiliani reagent (1) to each beaker and mix thoroughly. After ten minutes, determine the absorbances of the sample and standard at 590 mμ relative to the reagent blank at five-minute intervals. Record the maximum absorbance of the sample as *A*, and that of the standard as *S*. The quantity in mg. of total digitoxosides calculated as digoxin in the *Sample Preparation* is 1.25 *A*/*S*. The difference between this value and that obtained in the *Assay Procedure* indicates the quantity of other digitoxosides in the *Sample Preparation*.

Chromatographic Identification

Materials.—(*a*) *Immobile Solvent.*—Dilute 35 ml. of U. S. P. grade formamide to 100 ml. with acetone and mix. Prepare fresh weekly.

(*b*) *Trichloroacetic Acid Spray Reagent.*—Dissolve 6 Gm. of trichloroacetic acid in 25 ml. of U. S. P. chloroform and mix. Prepare fresh daily. Prior to use as a spray, add 0.2 ml. of 30% hydrogen peroxide per 10 ml. of reagent (2).

(*c*) *Mobile Solvent.*—Just prior to use, shake 100 ml. chloroform with 3 ml. redistilled formamide. Filter the chloroform layer through a cotton pledget.

(*d*) *Standard Preparation.*—Prepare a solution containing 2.5 mg. U. S. P. Reference Standard Digoxin per ml. of alcohol-chloroform (1 + 2).

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* [illegible] . . . [illegible] 1959, from the Department of [illegible] are, Food and Drug Adminis[illegible] [illegible] and Physical Sciences, Division [illegible] Washington, D. C.

[1] [illegible] lite MB 1, analytical grade, indicator-free, was found satisfactory.

(*e*) *Sample Preparation.*—Transfer 50 ml. of the *Assay Preparation* to a 125-ml. Erlenmeyer flask and evaporate to approximately 1 ml. on the steam bath with the aid of a current of air. Transfer the solution to a small weighing bottle with the aid of chloroform, and evaporate to dryness. Dissolve the residue in 0.25 ml. of alcohol-chloroform (1 + 2).

Procedure.—Arrange a suitable chamber for ascending chromatography (3) using Whatman No. 1, 8 x 8 inch filter paper impregnated with the *Immobile Solvent.* Spot separately 0.01 ml. of the *Sample Preparation* and *Standard Preparation,* in duplicate. Place the paper in the chamber, and permit the chromatogram to develop until the *Mobile Solvent* has moved to within an inch of the top. Remove the paper from the chamber and heat at 90° in a current of air for fifteen minutes. Remove the paper, spray with *Trichloroacetic Acid Spray Reagent,* and again heat at 90° for ten minutes. Examine the chromatogram under an ultraviolet lamp. The sample shows a single fluorescent spot, corresponding to that of the standard. (In order of decreasing R_f values, digitoxin gives a yellow-orange fluorescent spot; gitoxin, a yellow to blue spot; digoxin a blue spot; diginatin (4) and lanatoside C, blue spots near the starting line.)

DISCUSSION

The proposed method of assay was applied to U. S. P. Reference Standard Digoxin; U. S. P. Reference Standard Digitoxin; commercial samples of gitoxin, desacetyllanatoside C, and lanatoside C; two simulated tablet mixtures containing 0.0985% and 0.125% digoxin, respectively; two simulated injection mixtures, one containing 50% glycerol and the other 50% propylene glycol, and twenty commercial digoxin preparations of various types. The more polar members of the lanatoside C series yielded colors similar to that of digoxin with the reagent but desacetyllanatoside C was completely removed, and lanatoside C partially removed in the extraction process. Digitoxin and gitoxin yielded red colors in the procedure. The absorbance of the color due to digitoxin was about $^1/_3$, and that of gitoxin about $^1/_{20}$ of the absorbance due to digoxin at 620 mμ. Excessive quantities of these substances would be detected in the Keller-Kiliani assay and chromatographic test.

Analytical data obtained for U. S. P. Reference Standard Digoxin after extraction were identical with the values obtained for the unextracted standard in both colorimetric procedures. Recoveries of digoxin from simulated injections and tablet mixtures ranged from 97.4 to 100.5% of the quantities incorporated, and agreement between analysts and between replicate analyses demonstrated excellent reproducibility. It was necessary to use glassware scrupulously free of acid and base in performing the dinitrobenzene colorimetric test, since the alkalinity of the reaction mixture is critical. The ion exchange resin was introduced to eliminate interferences caused by stearate lubricants.

Assay values for digoxin and total digitoxosides in twenty commercial preparations are listed in

TABLE I.—ASSAYS OF COMMERCIAL DIGOXIN PREPARATIONS

		Per Cent of Declared		
		Digoxin by *m*-Dinitrobenzene		Digitoxosides by Keller-Kiliani Test
Sample	Description	Analyst 1	Analyst 2	
1	Powder	100.1	99.0	100.3
2	Powder	95.4	94.0	100.5
3	Powder	95.4	94.4	98.0
4	Powder	94.0	..	..
5	Injection, 0.25 mg./ml.	94.0	94.1	..
6	Injection, 0.25 mg./ml.	91.8	91.9	..
7	Injection, 0.25 mg./ml.	91.4	92.0	..
8	Elixir, 0.05 mg./ml.	95.5	95.6	..
9	Tablets, 0.25 mg./tablet	99.5	99.8	101.0
10	Tablets, 0.25 mg./tablet	96.3	98.2	93.6
11	Tablets, 0.25 mg./tablet	95.2	97.8	95.4
12	Tablets, 0.5 mg./tablet	97.1	96.8	96.7
13	Tablets, 0.25 mg./tablet	94.0	96.2	93.1
14	Tablets, 0.25 mg./tablet	96.2	95.8	95.4
15	Tablets, 0.25 mg./tablet	95.2	95.2	95.6
16	Tablets, 0.25 mg./tablet	97.9	94.3	101.4
17	Tablets, 0.25 mg./tablet	90.5	90.3	92.5
18	Tablets, 0.25 mg./tablet	88.7	88.3	88.7
19	Tablets, 0.25 mg./tablet	86.2	89.6	85.2
20	Tablets, 0.25 mg./tablet	89.7	89.5	89.3

Table I. The greatest difference between the two values (sample 16) was 7%, but these analyses were performed on different subsamples of tablets. In some cases the Keller-Kiliani value was as much as 4% lower than the *m*-dinitrobenzene assay, but this discrepancy may merely reflect the limits of error of the methods. The paper chromatograms showed no excessive quantities of contaminating steroids in any of the digoxin preparations.

SUMMARY

Methods are proposed for the chemical identification and analysis of digoxin in commercial preparations. These methods have been applied successfully to crystalline powders, tablets, injections, and elixirs containing digoxin.

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The Effect of Mouthwashes on the Oral Flora*

By MORRIS OSTROLENK† and WILLIAM WEISS

A study has been made of the efficacy of several commercial mouthwashes representing as many classes of chemical agents. The method employed tends to represent, as closely as possible, the conditions of use. The selective antibacterial activity of the washes was measured in terms of "before" and "after" total bacterial counts, and the incidence of streptococci, staphylococci, fusoform bacteria, and lactobacilli. There is included a study of the length of time that elapses after the use of the mouthwashes before the number of bacteria in the oral cavity reaches the number present before washing. Data obtained from these studies indicate that each of the compounds significantly reduces the total bacterial count in the mouth, and that some deal selectively with microorganisms. Each mouthwash, however, leaves residues of active bacteria among those removed from the oral cavity by a rinse technique. It appears also that the effect of the compounds is of short duration.

THIS COMMUNICATION continues the report of the investigation that has been in progress for the purpose of developing specific *in vivo* bacteriological methods for the evaluation of mouthwashes (1, 2).

The data presented here deal with: (*a*) The numbers of several types of microorganisms that can be washed out of the mouth by a rinse technique, (*b*) the selective antibacterial activity of several oral "antiseptic" mouthwashes, and (*c*) the length of time that elapses after the use of "antiseptic" mouthwashes before the number of bacteria in the oral cavity reaches the number present before washing.

METHODS

Two experiments were undertaken for the purposes of this study. In the first, five commercial mouthwashes, designated *B*, *C*, *E*, *G*, and *H*, and a sterile physiological salt solution, *X*, used as a control, were tested. Six subjects participated in this experiment.

A subject was given, at hourly intervals, four 30-second rinses, referred to as washes 1, 2, 3, and 4. Each rinse consisted of a 20-cc. solution of 0.85% salt. Midway between the second and third rinses, he was given an oral rinse consisting of one of the antiseptic mouthwashes or of the control, another salt solution.

Following each rinse, the wash solutions were expectorated into sterile petri dishes, appropriately diluted, and immediately planted into six semidiagnostic culture media as follows: (*a*) semisolid thioglycollate shake agar tubes (2), incubated at 34° for forty-eight hours; (*b*) Chapman's streptococcus agar (3), incubated at 34° for forty-eight hours; (*c*) Chapman's staphylococcus agar (4), incubated at 37° for seventy-two hours; (*d*) Littman's yeast medium (5), incubated at 30° for ninety-six hours; (*e*) Spaulding-Rettger fusobacterium medium (6), incubated in anaerobic jars at 37° for ninety-six hours; and (*f*) Tittsler's lactobacillus medium (7),[1] incubated at 37° for seven days.

These media were tested successfully in preliminary studies undertaken to verify that each was selective for the group of microorganisms that it was designed to detect. Seventeen volunteer subjects participated in the preliminary studies. The oral cavity of each subject harbored the test organisms, in widely varying numbers, but in only six was yeast recovered.

Six of these 17 subjects were chosen for the experiment. The six included three, one of whom was yeast positive, with bacterial counts on thioglycollate agar of less than 100 million; and three, two of whom were yeast positive, with bacterial counts on thioglycollate agar of 150 million or more.

Each of the six subjects received the mouthwashes *B*, *C*, *E*, *G*, *H*, and *X* once a week for six weeks, in the pattern of a Latin square arrangement (2). This procedure was repeated during an additional six weeks.

The methods employed in the second experiment were essentially those used in the first with the following differences: (*a*) The antiseptic mouthwash (or control salt solution *X*) was given between the first and second hourly rinses instead of between the second and third; (*b*) four subjects participated and each received, on an average, two of eight mouthwashes, including the control, each week for eight weeks. Eight determinations were made on each compound. The mouthwashes employed, in addition to *X*, were *B*, *C*, *D*, *E*, *F*, *G*, and *H*. The mouthwashes offered the test subjects were selected at random, with the restriction that no subject received the same mouthwash twice in one week; and (*c*) the oral rinse solutions (washes 1, 2, 3, and 4) and the control *X* were planted, in duplicate, in thioglycollate shake agar tubes for total bacterial counts.

RESULTS

Table I gives the results obtained from washes 1 and 2 in the first experiment. Subjects 4, 5, and 6, chosen because their total bacterial counts on thioglycollate agar were in excess of 150 million, re-

* [illegible] 9, 1958, from the Division of Anti-[illegible] Administration, Department of [illegible]elfare, Washington, D. C.
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[1] Canned whole tomatoes, filtrate, 200 cc.; distilled water, 800 cc.; tryptone, 5 Gm.; tryptose, 5 Gm.; yeast extract, 5 Gm.; agar, 12.5 Gm.; sodium azide 1:10,000. Adjust to pH 7.0 with sodium hydroxide, readjust with lactic acid to a final pH of 5.0.

tained this characteristic for the duration of the twelve-week test period. Their average first-wash bacterial counts were 163,489, and 225 million, respectively. Subjects 1, 2, and 3, with initial total bacterial counts of less than 100 million, showed first-wash averages of 89, 56, and 76 million for the same period. In addition, the three subjects selected as yeast negative and the three chosen as yeast positive remained, respectively, negative and positive throughout the twelve weeks.

In general, except for lactobacilli, bacterial counts rose between the first and second washes. It has been pointed out that this phenomenon is to be expected (2). The subjects consumed a meal sometime prior to each test and then ate no solids during the four-hour testing period. Eating is a mechanical degerming process; bacterial populations tend to decline after meals and then to return gradually to premeal levels.

Table II lists the third and fourth wash counts, expressed as a per cent of the second wash count (made thirty minutes prior to the application of the test solution), for each of the test compounds. These numerical estimates measure the activity of each of the test products, thirty and ninety minutes after administration.

A statistical analysis of variance was made separately for each test compound, for each organism group, and for both the thirty and ninety-minute intervals in which the antibacterial activity of each compound was compared with the control *X*.

Each of the five test products reduced significantly ($p < 0.05$) the total bacterial counts at both time intervals, in comparison with the reduction brought about by the mechanical action of the control salt solution *X*. There were similar significant reductions for each product in the streptococci counts.

Only compounds *E* and *G* reduced significantly the staphylococcus counts at the thirty-minute period, as compared with the cleansing action of the control. Mouthwash *G* was the only compound with a demonstrable antistaphylococcic effect at the ninety-minute measurement.

Only mouthwash *B*, and only for the time period of thirty minutes, gave a significant reduction in the yeast counts. Compounds *H* and *C* showed significant antibacterial activity thirty minutes after use in the fusoform bacterial counts, with only *H* giving prolonged activity for the additional hour until the ninety-minute measurement. Only *G* and *C* significantly reduced the lactobacilli counts, and each only at the thirty-minute period.

There were, for each of the counts, differences, often statistically significant, among the activities of the various test compounds. A study of Table II will show the hierarchies of the compounds with respect to their activities in each measurement.

The second experiment was designed to determine the length of time before total bacterial counts returned to normal following the use of seven commercial mouthwashes and a physiological salt-solution control. Results are given in Fig. 1.

The experiment shows that the logarithms of the bacterial counts obtained one-half hour, one and one-half hours, and two and one-half hours after the application of the mouthwashes lie approximately on straight lines. Recovery rates, expressed as a per cent increase over the preceding hourly wash bacterial counts, were: *C*, 15%; *B*, 30%; *G*, 33%; *H*, 41%; *E*, 49%; *D*, 56%; *F*, 66%; and the control, *X*, 26%. That is, on the average, the bacterial counts increased each hour by these percentages.

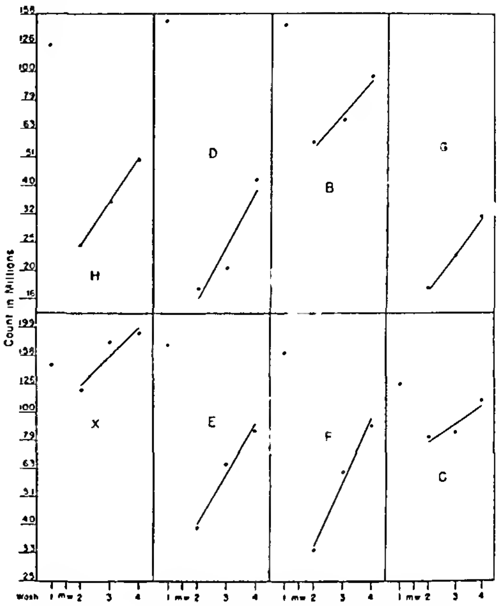

Fig. 1.—Duration of effect of test compounds.

On the basis of a statistical analysis, none of the recovery rates was significantly different from the others.

COMMENTS

In the evaluation of the *in vivo* antiseptic activity of mouthwashes, two measurements are of main significance. One of these is the count of microorganisms remaining in the oral cavity after the use of a compound, measured in terms of external recovery by some mechanical means. The technique employed here was an oral rinse.

It is evident that the five groups of microorganisms studied here occur at different numerical levels in the mouths of different individuals. Except for yeast, which has not been shown to vary appreciably with total bacterial counts, there is some variation in the population of group microorganisms related to the different total bacterial counts of individual subjects. On the basis of studies undertaken thus far (1, 2), in which approximately 200 subjects have participated, the total number of bacteria that can be removed from the mouth by rinsing ranges up to 500 million. No subject has yet been encountered, however, with a total count of less than 5 million following the use of an antiseptic mouthwash.

In the present study, the most effective product, *H*, permitted an average of 13.1% bacterial-survival thirty minutes after its use. Within an hour and one-half after the use of this product, the average number of bacteria recoverable by a rinse was 28% of what it had been prior to washing. At this point, there was an average of 40 million recoverable bacteria present in the mouths of the test subjects.

Product *H* significantly reduced at the thirty and ninety-minute levels the number of streptococci and

TABLE I.—AVERAGE NUMBER OF BACTERIA RECOVERED FROM FIRST AND SECOND WASHES

Wash	Total Count[a] 1	Total Count[a] 2	Streptococci[a] 1	Streptococci[a] 2	Staphylococci[b] 1	Staphylococci[b] 2	Yeast[b] 1	Yeast[b] 2	Fusobacteria[b] 1	Fusobacteria[b] 2	Lactobacilli[b] 1	Lactobacilli[b] 2
Subject 1	89	108	19	24	1.9	2.6	0	0	22.9	34.6	6.3	2.8
Subject 2	56	86	7	11	1.4	2.8	1.0	1.5	5.6	7.5	131.5	89.4
Subject 3	76	115	17	25	1.4	2.1	0	0	20.0	17.2	18.9	15.7
Subject 4	163	164	47	33	6.7	4.3	1.0	0.6	28.6	36.5	130.0	89.5
Subject 5	489	591	103	122	1.0	0.8	1.1	3.0	39.5	48.4	1840.0	2230.0
Subject 6	225	255	42	46	5.2	7.3	0	0	57.0	68.9	27.8	31.4
Average	137	173	28	33	2.2	2.7	1.0	1.4	23.4	28.6	68.5	54.0

[a] Counts in millions of bacteria [b] Counts in thousands of bacteria

TABLE II.—PER CENT RECOVERY OF MICROORGANISMS THIRTY AND NINETY MINUTES AFTER USE OF FIVE COMMERCIAL MOUTHWASHES AND A CONTROL

	Percentage[a] of Second Wash Counts											
	Total Count		Streptococci		Staphylococci		Yeast		Fusobacteria		Lactobacilli	
Compound	30 min	90 min	30 min	90 min	30 min	90 min	30 min.	90 min.	30 min.	90 min	30 min	90 min
B	24	52	32	52	58	62	21	47	36	92	58	51
C	34	44	18	34	40	65	45	44	25	45	17	26
E	19	31	9	19	20	78	46	43	54	132	41	68
G	18	23	6	9	23	31	52	51	110	111	13	48
H	13	28	11	24	76	87	30	36	11	16	23	61
X	81	107	105	118	72	104	82	97	69	105	89	57

[a] Rounded to nearest per cent

fusoform bacteria in the mouth, but in neither instance destroyed these microorganisms to an extent such that their rapid multiplication was significantly retarded. The product did not demonstrate significant antibacterial activity for the other groups of microorganisms. Although all of the five test products used in the first experiment significantly reduced the total number of bacteria in the oral cavity, no one compound showed significant reductions for all five bacterial groups.

The second, and probably more sensitive, measure of the antiseptic activity of the mouthwashes studied is the rapidity with which the bacterial flora of the oral cavity returns to its normal count. The results of the experiments undertaken here indicate that there is no long-lasting effect to be derived from the use of the products tested.

In the *in vivo* experiments described above, the methodology was that preferred by the authors. Judging by the literature, there are almost as many suggested test methods for the evaluation of liquid oral preparations as there are interested investigators. Unfortunately, the results obtained by this variety of methods are not comparable.

It would seem almost axiomatic that if liquid oral preparations are to be evaluated, some standardized *in vivo* techniques must be adopted. It has been suggested (2) that "for a practical approach, the method of obtaining oral-cavity samples for bacteriological analysis should be associated with the intended use of the class of products under test. It should be possible to correlate the efficacy of a medicated mouthwash with an innocuous oral rinse, toothpastes with brushing alone, germicidal gums with the chewing of sterile paraffin or some similar substance, and medicated lozenges with the corresponding effect of a placebo of equal volume and weight."

If the problem of measurement of the efficacy of any oral preparation merits consideration, it should not be too difficult for some interested body of investigators to settle on a test procedure acceptable to all.

SUMMARY

Data are given here on *in vivo* studies of the antibacterial activity of several commercial mouthwashes.

It appears from these data that each of these compounds significantly reduces the total bacterial count as measured by an oral rinse test method, and that some deal selectively with given groups of microorganisms.

The data indicate, however, that while some of the compounds produced a high kill of certain groups of microorganisms, none produced a complete or even near complete destruction of any group of microorganisms. It appears also that the effect of the mouthwashes under study is of short duration.

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Paper Chromatographic Evaluation of Menthol*

By LOLA V. HOPKINS and MARTIN I. BLAKE

A method is described for the conversion of menthol to a xanthate derivative which is isolated and treated with *p*-aminobenzaldehyde which produces a red color. The strong acidity of the color reagent regenerates menthol which is determined quantitatively by a paper chromatographic procedure which is described.

THE EXTENSIVE USE of menthol in the pharmaceutical industry has led to considerable interest in analytical methods for its determination. Several chromatographic procedures have been developed. Varma, Burt, and Schwarting (1) separated menthol from menthone by a column chromatographic method. Ito, Wakamatsu, and Kacuabara (2) used a chromatostrip technique to separate menthol from Japanese mint oil. The chromatograms were sprayed with aqueous vanillin and concentrated sulfuric acid to identify the menthol spots. Hamarneh, Blake, and Miller (3) reported the separation of menthol from peppermint oil by column chromatography and the quantitative determination of menthol content by a colorimetric method.

The formation of xanthates for identification of alcohols is suggested by Guenther (4). Kariyone, Hashimoto, and Kimura (5) separated xanthates of lower molecular weight alcohols by paper chromatography.

This investigation deals with the formation of the xanthate of menthol (a secondary alcohol), and its separation from other constituents in complex mixtures by paper chromatography. The xanthate is isolated and treated with *p*-dimethylaminobenzaldehyde test solution which produces a red color that can be used to determine the menthol content quantitatively. The strong acidity of the color reagent regenerates the free alcohol from the xanthate combination; a property of xanthates noted by Feigl (6). The color reaction was first observed by Masamune (7) who determined menthol colorimetrically in tissues and blood. The reaction has been applied by Hamarneh, Blake, and Miller (3) to the estimation of menthol in peppermint oil.

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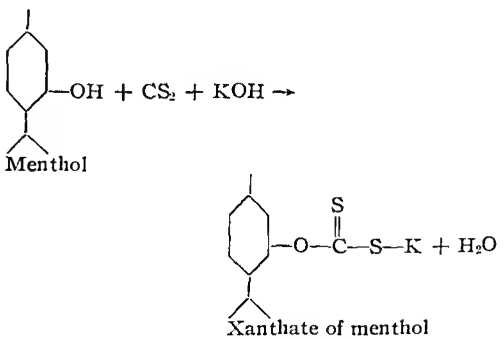
Abstracted from a thesis submitted to the Graduate School of the North Dakota Agricultural College in partial fulfillment of the requirements for the degree of Master of Science.

EXPERIMENTAL

Apparatus and Reagents.—Aloe chromatographic chamber, 12 x 24 inches; Aloe ultra-micro pipets, 2, 4, 6, 8, 10 λ; Klett-Summerson colorimeter, with green filter No. 54 (500 to 570 mμ); light source: ultraviolet lamp, type 7420, Hanovia Chemical and Manufacturing Co., Newark, N. J.; glass-stoppered graduated cylinders, 50 ml.; Whatman No. 1 filter paper for chromatography, 4 cm. wide, in a continuous roll; menthol, racemic, U.S.P., Magnus, Mabee, and Reynard; chloroform, reagent grade, Merck; carbon disulfide, reagent grade, Merck; Drierite, W. A. Hammond Drierite Co., Xenia, Ohio; *p*-dimethylaminobenzaldehyde test solution, U.S.P. XV, prepared daily; developing solvent: toluene:*n*-propanol (1:1) prepared daily from toluene, reagent grade, Merck, and *n*-propanol, reagent grade, Fisher Scientific.

Procedure.—Ascending chromatographic technique was used. The chamber was saturated with toluene for twenty-four hours before chromatographing by placing an open beaker of toluene in the chamber. Drierite was also placed in the chamber during this period to adsorb water vapor. The trough containing the developing solvent was placed in the chamber prior to use.

The sample was prepared for chromatograming by forming a xanthate. One milliliter of the sample was transferred by pipet to a 10-ml. test tube containing 0.5 ml. carbon disulfide (delivered by pipet) and about 0.1 Gm. (two pellets) of potassium hydroxide. The test tube was shaken intermittently for exactly ten minutes. The reaction proceeded according to the following equation:



The Whatman filter paper was cut in strips 50 cm. long. The end of the strip was cut to a point. The xanthate of menthol was applied to the paper by ultra-micro pipet at a position 5 cm. from the pointed end, and equidistant from the edges of the paper. The spot was made as small as possible with a diameter not more than 0.5 cm. Ultra-micro pipets were used for spot application to facilitate the application of a definite quantity of sample to the paper.

After the spot was air-dried, the paper was

placed in the chromatographic chamber, the pointed end just dipping into the developing solvent. This promoted even distribution of the developing solvent during chromatograming. Drierite remained in the chamber during development to adsorb water vapor. Optimum development time was two hours.

In the post-development procedure, the paper was removed from the chamber and air-dried at room temperature. The xanthate of menthol was located by its yellow luminescence under ultraviolet light. A section containing the xanthate was cut from the chromatogram and placed in a 50-ml. graduated cylinder. One milliliter of chloroform and 5 ml. of *p*-dimethylaminobenzaldehyde test solution were added from a buret. The cylinder was stoppered, then shaken briefly by hand to ensure complete mixing of the contents, and set aside for two hours for optimum color development. Because of the strong acidity of the color reagent, the paper dissolved and the xanthate of menthol was converted to free menthol. The menthol and the *p*-dimethylaminobenzaldehyde test solution produced a red color which was used to determine quantitatively the menthol content.

The contents of the glass-stoppered cylinder were transferred to a Klett-Summerson colorimeter tube. A blank was prepared from a section of Whatman No. 1 filter paper treated in the same manner as the sample and equal in size to the section containing the chromatogramed sample. The scale pointer of the Klett-Summerson colorimeter was adjusted to the zero marking with the blank. The light transmittance of the chromatogramed sample was then measured.

Preparation of the Standard Curve. Racemic menthol was used in the preparation of the standard curve. Exactly 1.7500 Gm. of menthol was weighed into a 10-ml. volumetric flask, and chloroform was added to the mark. This represented a concentration of 175 mg. of menthol per ml. of solution. One milliliter of this solution was used for preparing the xanthate derivative. Since the total volume obtained in the formation of the xanthate was 1.5 ml., a dilution factor of 3:2 was involved in all calculations. Thus, after xanthate formation, the concentration of menthol was $^2/_3 \times 175$ mg. or 116.6 mg./ml. Furthermore, since λ aliquots of the xanthate were used for chromatograming, a further dilution factor was necessary. A 1-λ aliquot contained 0.117 mg. of menthol. Concentrations of menthol from 0.040 mg. to 0.933 mg. were chromatogramed by varying the concentrations of the original menthol solutions and varying the size of the λ aliquots. A standard curve was constructed by plotting scale readings *versus* menthol concentration. The concentration range of 0.120 mg. to 0.700 mg. was found to obey Beer's law.

Application of Method.—Menthol samples were prepared for use as unknowns and were analyzed by the proposed procedure. Menthol concentrations were also determined in the presence of thymol and camphor. The data are reported in Table I.

TABLE I.—ANALYSIS OF MENTHOL SAMPLES

No.	Type Sample	Menthol Concentration, mg./ml. Taken[a]	Found[b]	Recovery, %
1	Menthol	160	159	99.37
2	Menthol	180	180	100.00
			179	99.44
3	Menthol	250	248	99.20
4	Menthol and camphor	150	154	102.66
5	Menthol and thymol	300	307	102.33
			295	98.33
6	Menthol, thymol, and camphor	150	147	98.00
7	Menthol, thymol, and camphor	550	547	99.45

[a] Concentration of original unknown solution
[b] Average of three determinations.

EXPERIMENTAL VARIABLES

R_f Values.—For the toluene:*n*-propanol developing solvent, the R_f for menthol as the xanthate was 0.97. The R_f for thymol was found to be 0.02, and for camphor 0.70. Pure samples of thymol and camphor were chromatogramed without pretreatment (as in xanthate formation of the menthol) to establish R_f values. Thymol and camphor were also chromatogramed with pretreatment as in xanthate formation. The R_f values were identical. Thymol was located by dipping the developed chromatogram in half-strength iodine and potassium iodide test solution (U.S.P. XV). A brown spot appeared in about twenty minutes. Camphor was located by dipping the developed chromatogram in a 0.4% solution of 2,4-dinitrophenylhydrazine in 1.5 *N* hydrochloric acid, followed by dipping in a 10% aqueous solution of sodium hydroxide. A red-brown color developed in a few minutes. This identification procedure was a modification of the method used by Rice, Keller, and Kirchner (8). Separation of menthol from thymol and camphor was readily effected.

Developing Solvent.—After considerable experimentation, the toluene:*n*-propanol (1:1) developing solvent was found to be satisfactory. Other solvents which were examined included ether, acetic acid, chloroform, *n*-butanol, isoamyl alcohol, carbon tetrachloride, *n*-hexanol, dioxane, *o*-xylene, morpholine, benzene, water, and combinations of these.

Development Time.—Optimum development time for chromatograming was found to be two hours. For shorter periods of time, the separation of constituents was poor. Prolonged time (over three hours) gave erratic results.

Color Development.—The length of time for optimum color development of the xanthate with the color reagent was two hours. Since the color reagent is extremely sensitive to menthol, it was necessary to prepare the color reagent in a room separate from the one used for preparation of samples. Fresh color reagent was prepared daily.

Temperature.—For all determinations, the temperature during chromatograming did not vary more than 5°. It was found that temperatures above 30° produced erratic results.

Critical Volume.—Clayton (9) has recommended that the amount of developing solvent used for a specific size chamber should be reported in all chromatographic work. In this study, it was found that 40 ml. of developing solvent was optimum for the 12 x 24 inch chamber.

Number of Papers in Chamber.—The simultaneous chromatograming of more than six papers in this chamber produced erratic results.

Water Vapor.—The presence of water vapor in the chamber resulted in a spreading of the xanthate of menthol along the entire length of the chromatogram. Therefore, Drierite was placed in the chamber during the saturation period and during chromatograming.

Spot Placement.—The positioning of the spot on the chromatographic paper is critical. If it was placed too close to the end of the paper, there was some loss of the spot producing low values. If the spot covered too large an area, low results were obtained.

DISCUSSION

Menthol is commonly used in pharmacy and medicine in combination with thymol and camphor as well as in the form of peppermint oil. An assay is officially recognized in the U.S.P. XV for the determination of total menthol in peppermint oil. There is no suitable method for estimating menthol in combination with thymol and camphor.

Paper chromatography was investigated as an analytical method because of its ease and simplicity. The equipment necessary is relatively inexpensive and the reagents are common laboratory chemicals. In the first phase of the proposed method, menthol is isolated from other constituents by an ascending paper chromatographic separation.

In the second phase of the method the chromatogramed sample is treated with *p*-dimethylaminobenzaldehyde test solution. The intensity of the red color which develops is dependent upon the menthol concentration. The light transmittance is measured with a Klett-Summerson colorimeter. The menthol concentration is determined from a standard curve.

While the basic chromatographic procedure is relatively simple, caution must be used to eliminate as many variables as possible. Complete saturation of the chamber is necessary for consistent results. One of the greatest difficulties is the variation in results due to high temperatures. For accurate application of a definite quantity of sample ultramicro pipets must be used. It is especially important to remove excess sample from the outside of the pipet before applying the sample to the chromatogram.

It is important that the developing solvent be completely removed from the developed chromatogram before reacting the xanthate with the color reagent. The developing solvent reacts with the color reagent to produce high results. The use of ground-glass stoppered cylinders during the color development is necessary to prevent volatization of the reagents.

The xanthate derivative has the advantage of reducing the volatility of menthol during chromatograming yet permits the development of a color reaction with the *p*-dimethylaminobenzaldehyde test solution. The red color which develops is used for the colorimetric determination of menthol concentration. The green filter No. 54 was employed in the Klett-Summerson colorimeter after experimentation showed that the maximum absorption occurred in the 500 to 570 mμ range.

The procedure is applicable to all concentrations of menthol. When the menthol concentration is above 50%, it is recommended that the sample be diluted prior to xanthate formation in order that the chromatogramed sample fall within the range of the standard curve.

The results of this investigation suggest a simple procedure for the determination of menthol, free and in the presence of thymol and camphor. Work is in progress to apply the method to the analysis of peppermint oil.

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Neurotoxicity of Hydrogen Cyanide*

By SEYMOUR LEVINE and BENJAMIN WEINSTEIN

Instead of exposing animals to a constant concentration of hydrogen cyanide, the concentration was deliberately varied according to the condition of the animals so as to produce a steady sublethal degree of intoxication. Only in this way was it possible to produce brain lesions in a regular and reproducible manner.

THE HISTOPATHOLOGIC LESIONS induced in experimental animals by toxic vapors are important in localizing susceptible tissues or cells and in determining the mechanism of toxic action. The purpose of this investigation was to devise a technique for administration of hydrogen cyanide that would produce lesions in simple, rapid, and reproducible fashion. The problem was approached by exposing rats to constant or variable concentrations of hydrogen cyanide for various periods of time under continuous observation and sacrificing the animals, after an interval, for histopathologic study. The technique which has been developed may be applicable to the study of other noxious vapors.

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EXPERIMENTAL

Apparatus.—Groups of rats were exposed in a 20-liter jar The hydrogen cyanide was generated by bubbling air through a solution of 1 to 5% potassium cyanide contained in a gas washing bottle with fritted glass disk. The hydrogen cyanide vapor was mixed with plain air in the desired proportions.

Single rats were exposed individually in 1-quart wide-mouth jars or fishbowls closed by two-hole number 14 rubber stoppers. Each stopper was fitted with an inflow Y-tube which combined the hydrogen cyanide vapor with plain air, and a straight tube for outflow (Fig. 1). Six such units were operated simultaneously with a single flowmeter and a single pump whose outflow was split into 6 parts by a glass manifold (Carrel-Dakin connecting tube). In order to permit separate control of the cyanide concentration in each unit, the air coming from each manifold port was further subdivided by a Y-tube. One arm of each Y conducted plain air directly to the corresponding jar, while the other arm led to the jar by way of a small individual fritted glass bubbler in a side-arm test tube containing potassium cyanide. Needle valve (or screw clamp) B controlled the proportion of flow in each arm, and hence the concentration of hydrogen cyanide in the jar.

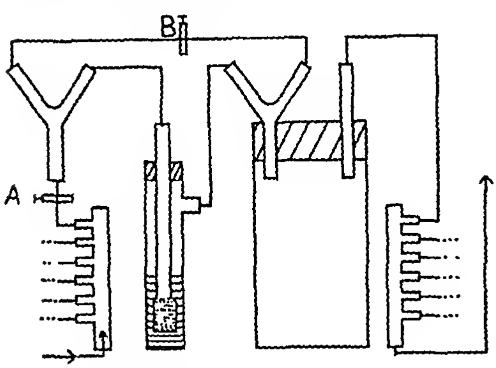

Fig. 1.—Diagram of one of the six units of the vapor expesure apparatus.

The six screw clamps A attached to the orifices of the manifold were essential for maintaining constant total flow at predetermined levels through each unit. They were partly closed so as to offer higher air resistance than the bubblers. Thus they assured a constant and equal flow through each chamber despite changes in resistance distally caused by opening and closing needle valves B during the exposure. In the absence of this arrangement the air flow from the pump would follow the path of least resistance through any of the valves B that happened to be open, rather than go through the bubblers with their inherent resistance Before exposures, the flowmeter was temporarily removed from the pump and used to measure the outflow from each unit which was equalized by slight adjustments of screw clamps A

Exposure to Constant Concentrations.—Adult albino rats exposed to hydrogen cyanide vapor of constant concentration became active after a few moments with increasingly violent running, jumping, and digging motions. Then there were hyperpnea, partial loss of postural tonus, convulsive twitches of extremities or tail, and occasional ineffectual running or jumping motions. The hyperpnea subsided as the rat lost its postural tonus completely and sank to the bottom of the jar. Slight movements brought on by tilting the jar soon disappeared, followed by loss of the ear twitch response to a brisk tap on the jar. Then there was progressive diminution in respiratory rate and depth until death occurred. No histopathologic lesions were detected in animals that died in this manner, presumably because there was insufficient time for histologic changes to develop.

In order to have a survival time sufficient to allow the development of lesions, lower but constant concentrations were used. If the concentration was sufficiently low, consciousness was never lost, but again, no lesions were produced even after exposures of many hours.

At intermediate concentration levels, consciousness was maintained for a while, sometimes for an hour, followed by sudden unconsciousness and death. This observation made it clear that a constant concentration of hydrogen cyanide did not mean constant experimental conditions, since the animal itself changed during such an exposure. Therefore, further attempts to produce lesions were based on keeping the rat at a constant physiological level by varying the cyanide concentration as needed.

Exposure of Groups to Varying Concentrations.—A group of eight female 250-Gm. rats received a single exposure of five to fifteen hours duration. The inflow of cyanide was reduced when many of the rats became unconscious and it was increased when some of them revived. Marked individual variation was noted so, that at any given moment, some rats were in terminal condition while others were conscious and capable of walking. Two rats died during the exposure. Of the six survivors sacrificed after forty-eight hours, 2 had brain lesions.

Ten male rats were subjected to 1 to 6 repeated exposures of five to nine hours each and 4 developed brain lesions. Five female rats were subjected to 3 to 9 similar prolonged exposures and 4 of this group developed brain lesions. In both these groups, some of the negative results were in animals who had received the maximum number of exposures.

The improved results, as compared to experiments with constant concentrations, were attributed to the nearly lethal intoxication maintained for a sufficiently prolonged time. However, it was inconvenient to conduct prolonged, multiple exposures, and there was great individual variation in susceptibility.

Exposure of Individual Rats to Varying Concentrations.—Female 250-Gm rats were exposed in individual jars, and six units were manipulated simultaneously. The rats were kept unconscious but when there was twitching, hyperpnea, or response to tilting the jar, the cyanide was increased When tapping the jar no longer elicited an ear twitch and when there was respiratory depression, the cyanide was decreased or discontinued. Individual variations were no longer a problem as each rat was exposed separately. A fairly even plane of sublethal intoxication was maintained for twenty to forty-five minutes. Longer exposures caused great mortality. Shorter exposures were sometimes ineffective in producing lesions. In 8 experiments involving 48 rats exposed under the optimum conditions described, 15 died during exposure and all 33 of the survivors had brain lesions.

In other experiments a somewhat milder but more prolonged and repeated intoxication was produced. Six rats were kept unconscious but with persistent muscular twitches, intermittent hyperpnea, and partial arousals for periods of two hours, repeated from 4 to 6 times. Only one rat showed a brain lesion, and this was slight.

These results indicated that a single, relatively brief exposure to hydrogen cyanide caused brain lesions in very high incidence, but only if the intoxication was maintained at a nearly lethal stage. The superiority of individual over group exposures, despite greatly shortened exposure time, was attributed to the maintenance of a constant degree of sublethal intoxication.

DISCUSSION

The optimum conditions for producing brain lesions with hydrogen cyanide were not provided by the administration of constant concentration of vapor. It was found necessary to induce a nearly lethal degree of intoxication, maintain that state for twenty minutes or more, and then permit the animal to survive long enough for lesions to develop. This could be accomplished only by varying the concentration of vapor in accordance with the condition of the animal as ascertained by constant inspection. Group exposures were unsuitable for this delicate control because of individual variations. Individual exposure in small separate jars provided a technique for producing brain lesions in high incidence (the histopathology, distribution, and pathogenesis of the lesions are discussed in another publication (1)).

These considerations may be applicable to noxious gases and vapors other than hydrogen cyanide. It is apparent that the absence of histopathologic lesions following a gas or vapor exposure may have little meaning unless the exposure was conducted under optimum conditions for the production of lesions.

SUMMARY

A technique for hydrogen cyanide administration has been described which induced a high incidence of brain lesions in rats. The rats were exposed in individual jars and maintained in a nearly lethal degree of intoxication by varying the concentration according to the condition of the animals. A simple apparatus for simultaneous operation of six individually controlled units with a single pump and flowmeter has been described.

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A Comprehensive Pharmaceutical Stability Testing Laboratory I*

Physical Layout of Laboratory and Facilities Available For Stability Testing

By LEON LACHMAN and JACK COOPER

This report illustrates the physical layout as well as the fixed equipment in a well organized stability laboratory in the pharmaceutical industry. It discusses the application of the equipment for testing pharmaceutical formulations as to their stability under varying conditions of temperature, humidity, and light intensity. Constant temperature equipment described consist of specially designed walk-in rooms as well as smaller size cabinets providing for a range of temperatures from −20 to 100°. In order to study the effect of varying humidity conditions on pharmaceutical products, constant humidity cabinets of a new design are illustrated and their operation discussed. These cabinets provide for a range of humidities from 30% relative humidity to 90% relative humidity. The influence of light on the stability of pharmaceuticals is discussed, and a specially fabricated light stability cabinet is described which simulates ordinary lighting found in the home, on a pharmacy's shelves, or in storage areas, but under exaggerated light intensity.

Although it has long been recognized by reliable pharmaceutical manufacturers that the potency of a drug is most important at the time it is administered to the patient, the exhaustive testing of new pharmaceutical preparations for physical and chemical stability is a procedure of relatively recent origin. Even today, not all pharmaceutical manufacturers perform extensive stability tests on new formulations. This may be due to the lack of trained personnel to plan and interpret the data resulting from such studies or to the absence of the essential equipment and comprehensive program needed to obtain pertinent and accurate stability data.

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Pharmaceutical manufacturers are becoming increasingly conscious of the absolute necessity of organizing a comprehensive stability testing program which provides adequate facilities for a skilled staff. In addition to the economic hazards involved in the introduction of an unstable product, the effects on the general scientific reputation of a manufacturer may be disastrous. With increasing competition in the pharmaceutical field, it is of considerable advantage for a new product to appear on the market as soon as possible after its formulation. However, in order to accomplish this, the manufacturer must first submit a New Drug Application to the Food and Drug Administration which, on the basis of adequate and accurate stability data, demonstrates the maintenance of labeled potency for a reasonable period of time.

The classical method usually employed to determine the stability of a new product is to expose the product to ordinary storage conditions (room temperature) for the period of time that the product would generally be stored in the normal market, e. g., anywhere from two to five years for most pharmaceuticals. An obvious disadvantage of such a procedure is the loss of time before marketing as well as the possible loss of market priority on a competitive product. Furthermore, if the preparation studied by this method appears to be unstable, there is no assurance without a similar time-consuming study that a modified product would not also be unstable.

In recent years, however, accelerated studies of stability have been employed as guides for the estimation of shelf life at ordinary shelf temperatures. Blythe (1) in 1954 made a survey of the stability testing programs of 40 pharmaceutical companies. He found that the greater number of companies use exaggerated conditions of temperature, humidity, and light to test the stability of new formulations. However, from the results presented in his report, it is clearly evident that the correlations between accelerated data to shelf storage conditions varied considerably due to different interpretations by manufacturers. This may be due to the fact that the correlations were intuitive, based on insufficient number of elevated storage conditions, or on empirical relations found in supposedly similar preparations.

Predictions of shelf life from accelerated stability studies can, however, be placed on a quantitative basis by the application of certain fundamental physicochemical principles. Studies of this type have recently been reported on in the literature (2–4), and they indicate that the shelf stability of products can be predicted within a relatively short period of time through the use of chemical kinetics. This method is applicable to simple pharmaceutical formulations as well as to more complex formulations containing several active ingredients, colorants, excipients, and the like.

It is the purpose of this report to present the design and facilities for the comprehensive stability testing program currently in use at Ciba Pharmaceutical Products, Inc. The physical plant and equipment for the Stability Laboratory used to test new formulations under exaggerated and ordinary storage conditions will be described. Through the use of this equipment and the application of certain physicochemical laws to the data obtained at the various storage conditions, it has been found that the shelf stability of new formulations can be predicted in a relatively short period of time and with a fair degree of accuracy.

PHYSICAL LAYOUT AND EQUIPMENT FOR THE STABILITY LABORATORY

The equipment for this laboratory has been chosen to allow for as diverse and varied conditions of testing pharmaceutical formulations as is feasible and necessary. With this equipment, stability tests for various formulations can readily be carried out under exaggerated conditions of temperature, humidity, and light intensity.

The range of temperature made available by the above equipment permits the testing of samples at temperatures ranging from −20 to 100°. There are three walk-in rooms set at 6°, 40°, and 50°, four laboratory size ovens set at anywhere from 60 to 100°, and one deep freeze cabinet set at −20°.

For testing formulations under varying conditions of humidity, seven cabinets are available, each one set at a different relative humidity. The range of humidities covered by these cabinets runs from 30% relative humidity to 90% relative humidity in increments of 10% relative humidity at 25°. However, although these cabinets are set at 25°, they can be changed to different temperatures and different relative humidities.

In order to determine the stability of formulations with respect to light, a special cabinet has been fabricated according to our specifications. This cabinet simulates ordinary lighting found on a pharmacy's shelves, in the home, or in various other storage areas for pharmaceuticals, but under accelerated light intensity.

This report will contain only a description of the fixed equipment for the stability laboratory. An overall drawing of the equipment and layout of the laboratory is presented in Fig. 1.

In order to present a clearer picture of the various pieces of equipment in this laboratory, it seems advisable to break down the equipment as follows, and discuss each piece of equipment in greater detail under these classifications: constant temperature equip-

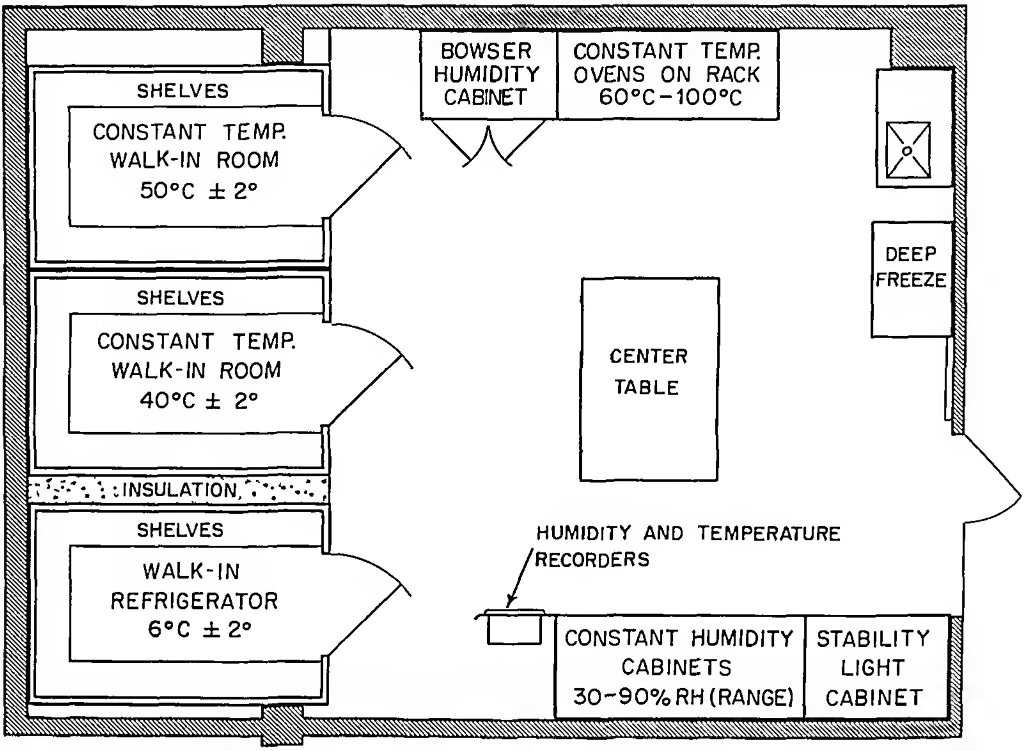

Fig 1.—An over-all drawing of the equipment and layout for the stability laboratory.

ment, constant humidity equipment, light stability cabinet, and humidity and temperature sensing elements and recorders

Constant Temperature Equipment.—There are three Electric Hotpack walk-in constant temperature rooms in the laboratory set at 6, 40, and 50° A description of these rooms is presented in Fig 2. The rooms illustrated in this figure have been manufactured according to our specifications and they have been designed to appear as one unit along one of the walls of the stability laboratory. These rooms are constructed of heavy 16 gauge aluminum throughout, and insulated with 3 inch precompressed glass wool Glass wool was chosen as the insulating medium since it has low thermal activity and because it is nonhygroscopic, fireproof, and unharmed by corrosive fumes

The interior dimensions of these rooms are 6 feet wide by 8 feet deep by 7 feet high. There are six tiers of 10-inch wide shelving on three sides of the room for storage of samples. These shelves are adjustable on 2-inch centers, and any or all of them can be removed if desired.

For the rooms to be kept at 40° and 50°, scientifically designed heaters and air circulation maintain a constant temperature, controlled by an adjustable mercury thermostat with one change of air every 10 minutes. The heaters consist of nichrome coils running through porcelain refractories, scientifically distributed in louvered baffles, and mounted on the three sides of the room near the floor. The air is drawn into the chamber through two ports located at the lower front section of the chamber. The air then passes over the heater banks, is heated, and rises up through the louvers and into the work chamber of

Fig. 2.—A description of the walk-in constant temperature rooms maintained at 6, 40, and 50° ± 1.5°.

the unit. After transmitting its heat to the work load, the air then passes into the ventilating duct located in the ceiling of the room. At the front of the ventilating duct is a 6-inch exhaust fan that expels the used air outside the room. The thermostat is of the sealed adjustable mercury type. It is situated

on the rear wall of the room in such a position as to reflect accurately the control temperature fluctuations within the room The accuracy of the thermostat is protected against external vibrations by a sealed mercury relay that prevents arcing in the thermostat operation

As a safety control for temperature override, an adjustable, independent overtemperature thermal switch is built into the room to prevent the temperature of the room from exceeding the control temperature. It operates an alarm buzzer located on the front panel and the power supply to the heaters This control can be independently set to operate from 3 to 20° above the control thermostat, and will automatically take over the control of the heaters at this higher temperature as well as automatically turn on the alarm buzzer.

For the room to be kept at 6°, there is present a refrigeration system in addition to the heating units In this room the heated and cooled air is apportioned, resulting in a rapid, yet close control of the conditioning medium Two separate ducts convey the heated and cooled air to a centrifugal circulating blower. These ducts are opened and closed by automatic dampers which insure the correct quantity and correct proportions of conditioned air. The streams of air coming from these two ducts are blended by the circulation blower, and then passed through a duct to the diffusers in the ceiling of the room where the conditioned air is distributed uniformly throughout the interior of the chamber.

The temperature of the three rooms is adjusted so that there is only ± 0.5° differential at the thermostat and ±1.5° between widely located points on the top and bottom shelves.

A room adjacent to the stability laboratory is employed for room temperature (ambient) storage. This room has shelving along three of its walls to accommodate samples being stored under ambient conditions.

Since samples stored at temperatures of 60° and above will generally be kept at these temperatures for shorter periods of time than at the lower temperatures, and since not all preparations which are stored at the lower temperatures will also be stored at temperatures of 60° and above, it was felt that walk-in rooms were not required for these higher temperature conditions. Instead regular laboratory size ovens appear to be of adequate capacity for these higher temperature conditions of testing.

In order to accomplish this, four Thelco convection ovens were installed in the stability laboratory for storing of samples at temperatures of 60° and above. These ovens were placed into an angle frame so that they appear as one unit and are shown in Fig. 3 These ovens set at temperatures above 60° can also be of use in accelerated short-term studies which precede the comprehensive stability study in order to screen possible formulations for the one of maximum stability

These ovens have a thermostat sensitivity of ±0.25° and a temperature uniformity of ±1.1° A balanced $^{1}/_{20}$ hp turboblower with a system of baffles provides a smooth flow of air constantly circulated, exhibiting predetermined air flow patterns which result in a more even distribution of heated air over the entire working area.

The ovens are constructed of an aluminum interior and an enameled exterior All sides of the oven are

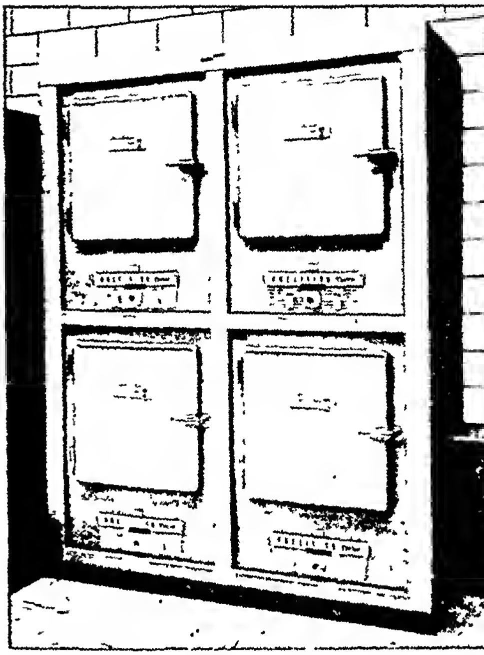

Fig 3.—Four laboratory size ovens placed into an angle frame and set at temperatures of 60° and higher ±1 1°.

insulated with glass wool for better insulating qualities The internal dimensions of the chamber are 18 inches wide by 14 inches deep by 19 inches high. Two adjustable shelves are supplied for each cabinet.

For samples stored at −20°, a Kelvinator deep freeze is employed. Its dimensions are 26 inches high by 34 inches long by 20 inches wide.

With the following constant temperature equipment just described, it is possible to test new formulations within a temperature range from −20 to 100°. From results obtained under accelerated temperature storage, one is able to predict the stability of new pharmaceuticals at ordinary shelf temperature by considering the temperature dependency of the reaction. The most satisfactory method for expressing the influence of temperature on the stability of an active constituent is the quantitative method of Arrhenius.

Constant Humidity Equipment.—In order to study the effect of varying conditions of humidity on certain pharmaceutical formulations undergoing stability testing, a six cabinet humidity installation has been fabricated for the stability laboratory by the Electric Hotpack Company. However, at the present time only four of the six cabinets are installed, but the framework for the additional two cabinets is included so that at a later date the additional two cabinets can be readily added. The overall assembly of the cabinets is presented in Fig. 4.

The relative humidities to be maintained by the four cabinets at 25° ±0.5° are 30, 60, 70, and 90% relative humidity ±3%.

All of the cabinets are constructed of stainless steel and so fabricated as to be watertight. The internal dimensions of each chamber are $19^{1}/_{2}$ inches wide by 18 inches deep by 20 inches high.

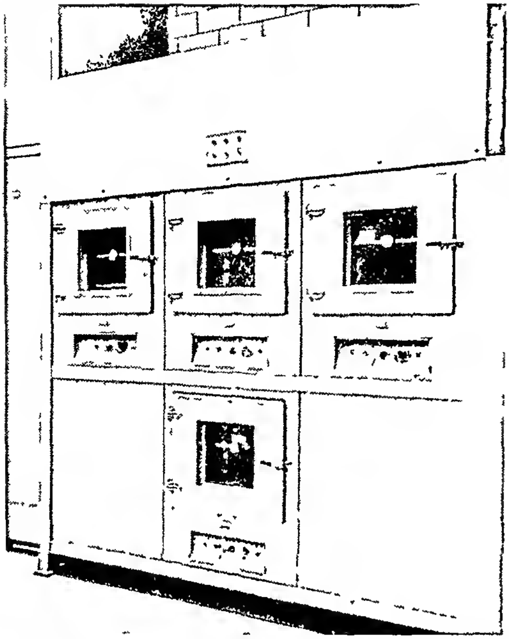

Fig 4—A six cabinet constant humidity installation with four cabinets set at 30, 60, 70, and 90% ±3% relative humidity

For the cabinets set at 60, 70, and 90% relative humidity, coolant coils, humidity nozzles, and temperature controls are required The water for the coolant coils comes from an insulated and accurately controlled refrigerated water bath mounted on top of the frame housing the humidity cabinets A motor driven stirrer agitates the water for precise temperature uniformity A built-in electric pump is provided for circulating the coolant water to each chamber The heaters in each chamber are controlled by an accurate hydraulic type thermostat. The on-off control of the heaters counteracting the constant coolant supply are intended to provide extremely close temperature control The air in the cabinets is circulated from the front to the rear and then passes over heaters and cooling coils located behind the rear and side wall baffles After conditioning, the air is expelled for recirculation again Water is passed into the cabinets through humidifier nozzles in order to obtain the desired relative humidity.

For the cabinet set at 30% relative humidity, a dehumidifier is used in place of the humidifier nozzle, to cause the low relative humidity. This dehumidifier consists of a Dryomatic air dryer mounted on top of the angle frame containing the humidity cabinets. The Dryomatic unit is completely self-contained and never needs replacement of desiccant material. Otherwise, the rest of the installation is similar to that of the other three cabinets

The heater controls for each cabinet are mounted in a recessed panel at the front of each cabinet, while the humidity is controlled by a Humidistat mounted in the chamber.

The services, the dehumidifier, and the refrigerated water bath are located on top of the angle frame containing the humidity cabinets and have been paneled off so that they are not visible.

In addition to the above humidity cabinets, a floor model Bowser humidity cabinet set at 80% relative humidity is available in the stability laboratory. This cabinet is an individual piece of equipment requiring its own services, water supply for the humidity tank, heater controls, and refrigeration Its internal dimensions are 36 inches wide by 20 inches deep by 43 inches high. An overall view of this unit is presented in Fig 5 Since this unit can be readily purchased and due to the fact there is sufficient manufacturer's literature available as to the temperature and humidity controls used in this unit, a discussion of same will therefore, be omitted.

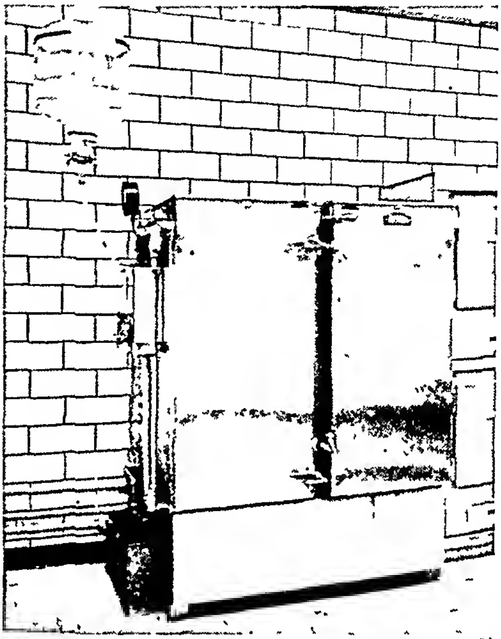

Fig. 5—A floor model humidity cabinet set at 80% ±3% relative humidity.

Light Stability Cabinet.—In order to determine the most suitable lighting system for use in observing the effect of light on a pharmaceutical formulation, a thorough investigation of the different equipment and techniques utilized for studies performed under accelerated light conditions was made. A few of the sources of light commonly employed to determine the stability of a product to light are direct sunlight, diffused light, sunlamps and other types of ultraviolet lamps, a fadeometer, and other light sources Since sun intensity varies from season to season and day to day, and since tests are usually conducted for uniform periods of time, it is difficult to quantitate the degree of exaggeration represented by sunlight. Arny, *et al* (5), in studying the stability of various chemicals felt that spring and fall sun, which would represent approximately the average for the year, represented 10 to 12 times the effect of north light. Furthermore, since as the intensity of the sunlight increases, the heat absorbed by the product increases, there may very well be a potentiation of the degradation due to heat besides that caused by the sunlight In addition, since products are usually stored in a pharmacy, in a storage warehouse, or in the home, the amount of ultraviolet rays entering from the sunlight coming through the glass store

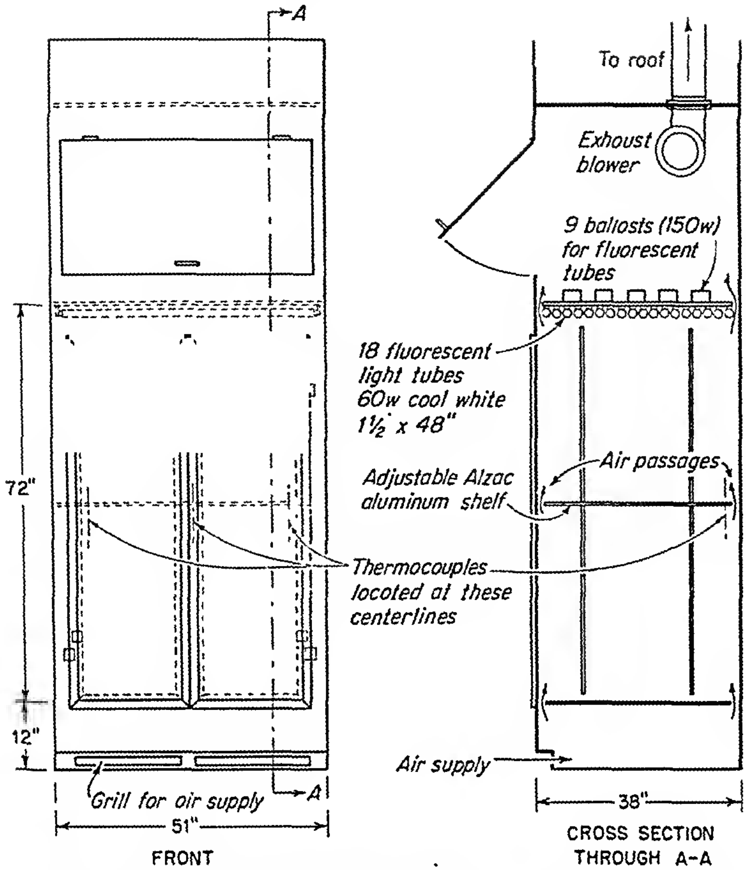

Fig. 6.—A comprehensive description of the lighting cabinet used for exaggerated light stability testing with its lighting equipment and specifications.

front or window panes of a home or a warehouse is less than 5%. Therefore, the sun ultraviolet rays would seemingly contribute very little, while light intensity should contribute significantly when studying the stability of pharmaceutical formulations with respect to light.

With the above in mind, a cabinet was designed to our specifications which contains a lighting system intended to simulate ordinary lighting found on a pharmacy's shelves, in the home, or in various other storage areas for pharmaceuticals, but under accelerated light intensity, e. g., 20 fold. The information obtained by exposing pharmaceutical formulations to this sort of accelerated lighting conditions should be extremely useful for predicting the stability of a formulation on a more quantitative basis when exposed to ordinary light conditions for an extended period of time. A later report will contain experimental data on the usefulness of this cabinet, but for the present only a detailed description of the cabinet will be given.

A comprehensive description of the lighting cabinet with its lighting equipment and specifications is presented in Fig. 6, and a photograph of the cabinet is presented in Fig. 7. It can be seen from Fig. 6 that the information contained therein is rather detailed and does not appear to require much explanation. The cabinet has been prepared according to the dimensions in Fig. 6 to cut down on light fall-off

Fig. 7.—A front view photograph of the light stability cabinet.

as much as possible. The walls were painted a flat white on the inside for reflectance purposes. The Alzac aluminum is utilized behind the lamp tubes and on the tray for light reflection purposes.

The fluorescent tubes used in this cabinet were chosen since they give off the least amount of heat per foot-candles of light intensity produced. Any heat given off by the fluorescent tubes is removed by a blower above the ballasts containing the tubes. The temperature of the shelf containing the products being tested is essentially ambient temperature, and is being constantly recorded through three thermocouple leads at the shelf attached to a Minneapolis-Honeywell 16 point Strip Chart Temperature Recorder.

The light intensity appears to be the essential factor which affects the stability of materials stored under ordinary conditions. The higher the intensity, the greater should be the effect on the material. The approximate foot-candles of intensity hitting the shelf at varying distances from the source of light can readily be calculated. For example, the approximate foot-candles of intensity available in this cabinet at a distance of three feet from the bottom can be calculated in the following manner:

$$L.\,L.^{1} = \frac{FTC \times \text{area}}{U.\,F. \times M.\,F.}$$

$$FTC = \frac{L.\,L. \times U.\,F. \times M.\,F.}{\text{area}}$$

$$FTC = \frac{3250 \text{ lumens} \times 18 \text{ lamps} \times 0.3 \times 0.75}{12 \text{ feet}} = 1100 \text{ foot-candles}$$

$$FTC = 1100 \text{ foot-candles}$$

Since the average level of light intensity in most areas where pharmaceuticals are usually stored is approximately 50 foot-candles, the lighting in the cabinet should give essentially a 20-fold increase in light intensity if the shelf containing the samples under study is three feet from the bottom of the cabinet.

Due the fact that the shelf in this cabinet is adjustable, it can be regulated to any height desired. This will enable the cabinet to be adjusted to give varying light intensities according to the following equation: Light intensity $= 1/d^2$, where d = distance (feet).

Humidity and Temperature Sensing Elements and Recorders.—It appeared desirous to have a constant recording of the temperatures in the various cabinets in the stability laboratory as well as the humidities in the constant humidity cabinets in order to be able to check out the temperatures or humidities in case of irregular assay results for samples stored in these cabinets. This has been accomplished by installing two Brown Strip Chart Recording Electronik Potentiometers on a panel as shown in Fig. 8.

The strip chart recorder for the temperatures is a 16 point recorder and was attached to the various cabinets through iron constantan extension wire, 16 gauge with weatherproof over polyvinyl insulation. The thermocouples employed are manufactured by the Minneapolis-Honeywell Regulator Company. One thermocouple is inserted into each of the ports of the temperature and humidity cabinets, while three thermocouples are attached at different points on the shelf of the light stability cabinet.

[1] *L. L.* = light lumens; *FTC* = foot-candle, *M. F.* = maintenance factor; *U. F.* = utilization factor = 0.3 of level.

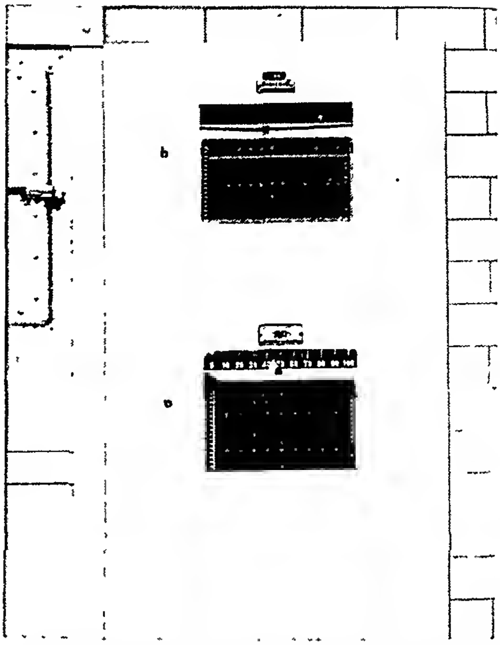

Fig. 8.—Two Brown Strip Chart Recording Electronik Potentiometers used to record temperature and humidity in the several stability cabinets and rooms.

The printing record on the temperature chart consists of a (+) and a number. The number and color of printing identifies the thermocouple. The chart is graduated from 0 to 125° which is the same as the scale.

The strip chart recorder for the humidities is similar to that for the temperatures, but is only of 12 points. Here humidity sensing elements manufactured by the American Instrument Company are employed. These sensing elements are inserted into their respective humidity cabinets through ports especially prepared for them and are attached to the recorder with the aid of sensing element extension cables.

The operation of these sensing elements is based on the ability of a hygroscopic film to change their electrical resistance instantly with micro changes in moisture content (relative humidity). These elements should detect changes as small as 0.15% relative humidity and measurements are reported to be accurate within ±1.5% relative humidity.

GENERAL DISCUSSION

The foregoing presentation was intended to present to the reader an overall picture of the physical layout of the Stability Laboratory of the Pharmacy Research and Development Division of Ciba Pharmaceutical Products, Inc., as well as the fixed equipment to be employed in this laboratory. The operation of the various equipment as well as their specifications were described briefly.

It appears that through the use of the facilities made available by this laboratory, it will be possible to predict the stability of new pharmaceutical formulations at ordinary shelf storage from accelerated testing data with a good degree of accuracy. This will in turn make it possible to market new pharmaceutical preparations with a definite degree of assurance as to the shelf life of the preparation and within as short a time after formulation as possible. This is becoming more and more essential due to the fact that competition in the pharmaceutical field has substantially increased within the past few years, and it is, therefore, advantageous for the pharmaceutical manufacturer to introduce on the market a new product as soon after formulation as is feasible.

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(3) Garrett, E. R., *ibid.*, 45, 171(1956).
(4) Garrett, E. R., *ibid.*, 45, 470(1956).
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A Comprehensive Pharmaceutical Stability Testing Laboratory II*

Record and Control System Employed for the Laboratory

By LEON LACHMAN and JACK COOPER

A description of a comprehensive record and control system employed as an integral part of the stability laboratory is presented. This is intended to facilitate the recording of the numerous data that become available from the products undergoing stability testing and to provide an accurate system for sending of samples for analysis at the correct time intervals. Stability evaluation cards used to record data of a product undergoing stability testing are described. Different color cards are employed for different storage conditions. The information preprinted on the cards varies according to the type of dosage form. The labels to be placed on the products being stored are shown to be similar in color to that of the stability evaluation cards. The captions preprinted on them are discussed. A simple foolproof control method for sampling preparations at designated time intervals is demonstrated. Simplified analysis report forms to be employed when samples are sent to the other groups for testing are illustrated and discussed. In order to have available a graphic picture of the progress for the various dosage forms being tested, the use of a visual flow chart is described.

IN THE PRECEDING PAPER of this series (1), the physical layout and available equipment of a comprehensive pharmaceutical stability testing laboratory were described. The variety of dosage forms as well as the diverse storage conditions to which they are exposed render the organization and control of this laboratory a complex function. Since time is the key factor in utilizing the data obtained from the operation of such a laboratory, the system for identification of samples, recording of experimental results and planning of correct test intervals is of the utmost importance. It is the purpose of this report to describe the carefully organized, detailed but practical record and control system for stability evaluation currently employed in our Pharmacy Research and Development Division. In operation for over a year, the value of this "bookkeeping" system to the whole stability testing program has been clearly demonstrated.

Essentially, this record and control system can be divided into the following five parts: I. stability evaluation cards, II. labeling of samples under study, III. sample "withdrawal for test" file, IV. analysis report forms, and V. visual flow chart of studies in progress.

In order to obtain a clear picture of the function of each of the several parts of the record and control system, each part will now be described in detail.

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Presented to the Scientific Section, A. PH. A., Los Angeles meeting, April, 1958.

RECORD AND CONTROL SYSTEM

I. Stability Evaluation Cards.—Since many formulations would be simultaneously under study, it was necessary to devise a recording system for handling the considerable volume of data in an orderly manner. A standard card 9 inches by 12 inches in size was found to be large enough to contain the required amount of information and yet of a size suitable for filing in an office file for legal documents. For each environmental condition of sample storage, a card of different color is used. Figure 1 represents the front of a representative card.

In examining the card, it can be seen that certain information is preprinted. For example, the Roman numeral in the right hand corner designates the classification of the formulation. This distinction is

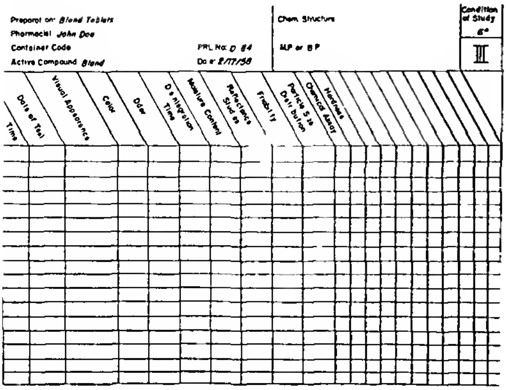

Fig. 1.—Front of a stability evaluation card.

based on certain similarities in optimal storage conditions for each classification and acts as a check on the scheduling of appropriate tests. The four classes used are:

A. Liquid Pharmaceutical Preparations (I).
B. Creams, Ointments, Jellies, and Suppositories (II).
C. Tablets, Powders, Capsules (III).
D. Lyophilized Preparations (IV).

Each classification is represented by the same color card for the same storage condition, but the preprinting of the tests to be performed will vary with the cards for each classification. However, even within a classification a particular physical or chemical test may not be applicable and should be omitted. Furthermore, there are blank spaces available for listing additional or special tests required by some formulations. Under various preprinted headings certain information concerning the active constituents will be entered prior to the actual initiation of the study.

Figure 2 shows the back of the card where provision has been made for listing the results of biological tests, the exact formula of the preparation under study and special comments of value. In scheduling a stability study, it is necessary to fill in the appropriate information on as many cards as there are storage conditions in advance of the actual initiation of the tests. These cards are then placed in a folder which bears the name of the product and the folder is placed in the stability file on the day the study is officially initiated. As the study progresses, the results of the various tests are listed on the cards A glance through the folder serves to inform a staff member as to the stability status of any product under study.

II. Labeling of Samples Under Study.—Since the labels on the samples under stability study match the color of the evaluation cards and the storage condition is preprinted in the upper right hand corner, good control of sample placement is achieved. Should a preparation intended for storage under one condition be placed in a cabinet maintaining a different condition, the label color would pointedly indicate the error. Figure 3 shows a representative label. The information on this label identifies the product, the classification, the research pharmacist, the date the study was initiated, and the pharmacist's research book number for the preparation.

III. Sample "Withdrawal for Test" File.—To avoid failure in withdrawal of samples at the appropriate testing time, a special warning file card system was devised. Figure 4 shows a sample 3 x 5 card of this type with its preprinted captions. Each drawer of the file cabinet holds the cards for one year with further subdivision into months and days.

When a stability study on a preparation is initiated, cards are prepared for each time interval of testing for the entire length of the study. It can be seen from Fig. 4 that the captions on the cards require notation of the date, the test or tests to be performed, the pharmacist's name, the name of the preparation, and the pharmacist's research book number of the preparation.

Once all the cards for the study are prepared, they are filed according to year, month, and day in the appropriate file on the day the study is officially initiated. When using this file, the technician pulls all the cards for that day and immediately begins to pull the required samples in anticipation of the tests to be performed on that day.

IV. Analysis Report Forms.—Since many of the tests for formulations under stability study are performed by divisions other than Pharmacy Research, it is necessary for these divisions to report the results obtained to the Pharmacy Research and Development Division Stability Laboratory as soon after their completion as possible. This should be done in order to keep the stability evaluation cards up to date so that the complete picture of the stability of the formulation is always available. However, in order to accomplish this, a good deal of paper work

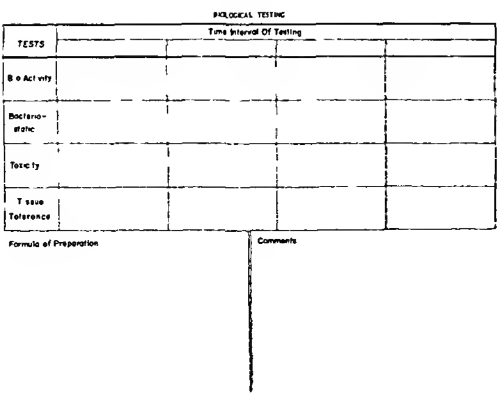

Fig. 2.—Back of a stability evaluation card.

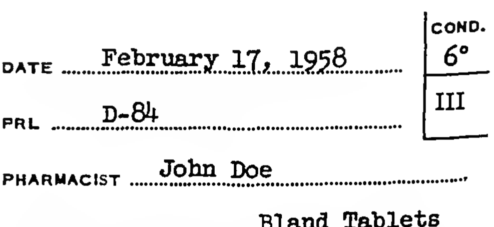

Fig. 3.—A representative label employed in the stability laboratory for samples undergoing stability testing.

DATE Feb. 17, 1958 PHARM. John Doe PRL# D-84

PREP. Bland Tablets

TESTS TO BE RUN: R.T.
40°
50°
60°
70% R.H.

Fig. 4.—"Withdrawal for test" file card.

by the cooperating divisions would be entailed if they had to write a memorandum each time they completed their tests on a formulation.

In order to substantially decrease this paper work and to facilitate reporting of data, Analysis Report Forms were prepared and a representative sample is shown in Fig. 5.

Before a sample is sent to a cooperating division for testing, these forms are filled out in triplicate and two forms are sent along with the sample to the division which will perform the tests and one copy is retained by the stability laboratory. Upon completion of the tests, the results are entered on both forms, the analyst's signature is affixed and one of the forms is returned to the stability laboratory for posting of the results on the stability evaluation cards. The completed form is filed after first removing the uncompleted form from the file. With

CIBA - Summit
Pharmacy Research and Development Division
Stability Laboratory

ANALYSIS REPORT FORM

TO: Analytical Research

SAMPLE: Bland Tablets

PRL NO.: D-84 DATE: Feb. 17, 1958

STORAGE COND.	ASSAY DESIRED	RESULTS
R.T.	chemical assay	
40°	chemical assay	
50°	chemical assay	
60°	chemical assay	

Analysis by:

Requested by: Leon Lachman 45010-X

Fig. 5.—A sample of a stability laboratory analysis report form.

the use of these forms, a running inventory is available with regards to analysis results still not completed by the cooperating divisions.

While on the topic of analysis by cooperating divisions, it appears advisable to mention that for a comprehensive stability testing program to be a success, the Pharmacy Research group must work in close cooperation with the Analytical and Biological groups in planning the tests for the various preparations to be studied. Usually, chemical, physicochemical, and physical assay methods can predict the stability of an active ingredient in a formulation. However, in certain instances, biological tests are more indicative of the stability of a formulation and are an important adjunct to a stability program.

V. Visual Flow Chart of Studies in Progress.—This chart is intended to give at a moment's notice the general overall status of a formulation undergoing stability study. The information made available by looking at this chart is the time interval of testing and the dates for the intervals, to what extent the study has progressed, whether a new drug application has been applied for, whether a new drug application has been approved or rejected, and at what point of the program the studies were discontinued and for what reason. The flow chart employed is manufactured by Broadmaster.[1] Through the use of different color cards which fit onto the chart, one is able to obtain a clear cut, graphic picture of the overall stability program that is in progress.

GENERAL DISCUSSION

In the previous portions of this report, the various aspects of the record and control system employed for the comprehensive stability testing program at Ciba Pharmaceutical Products, Inc., were described. It was shown how each part of the system contributes to the efficient functioning of the stability laboratory. Moreover, experience has shown this system to be an essential adjunct in the operation of this laboratory.

Through the use of the stability evaluation cards, it is possible to efficiently record all the stability data that becomes available for a new dosage form under study. By use of chemical kinetic equations and the available experimental data, it is possible to predict the stability of the product at ordinary shelf temperature by extrapolating the curve obtained from accelerated data.

Therefore, the overall effect of the equipment and the record and control system in the stability laboratory is to permit the laboratory to function efficiently as well as to make possible an orderly accumulation of sufficient and pertinent stability data. This in turn will permit the prediction of the stability of a new pharmaceutical formulation at ordinary shelf storage with a good degree of accuracy and in a relatively short period of time.

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[1] Graphic Systems, 55 West 42nd Street, New York, N. Y.

A Folic Acid Method Involving Permanganate Oxidation*

By S. S. SCHIAFFINO, J. M. WEBB†, H. W. LOY, and O. L. KLINE

A chemical method for the determination of folic acid in pharmaceutical products is presented. The method involves digesting the folic acid in a mildly alkaline, buffered solution (3% K_2HPO_4) followed by splitting with $KMnO_4$, diazotizing the resulting PABA, and coupling with the Bratton-Marshall reagent (*n*-1-naphthyl)-ethylene-diamine dihydrochloride). In the presence of extraneous colored substances, as may be found in some pharmaceutical preparations, the chromogenic compound is extracted into isobutyl alcohol with no diminution in intensity of color, or change in wavelength of maximal absorption (550 mμ). Ferrous compounds and ascorbic acid do not interfere in the method as they are found to do in the NH_4OH digestion-zinc reduction method. The assay time with the proposed method is less because of the shorter time required to cleave folic acid by oxidation. The results are reproducible, and good folic acid recoveries are obtained.

THE PRESENT U. S. P. chemical method (1) for determining folic acid is based on the reduction of folic acid with zinc to yield *p*-aminobenzoic acid (PABA) which is diazotized and coupled with the Bratton-Marshall reagent (2) to produce a pink color which can be measured spectrophotometrically. In the application of the method to the assay of folic acid alone, or folic acid contained in pharmaceutical preparations, several difficulties have been reported. Hutchings, *et al.* (3), reported that variable results were obtained, and stated that the addition of gelatin, as a protective colloid during the zinc dust reduction, was useful in assuring a quantitative yield of the amine. Later, Kaselis, *et al.* (4), reported that this "protective" action varied with the quality of gelatin used. They recommended the use of zinc amalgam as a reductant, stating that it gave more satisfactory results than zinc dust. Glazko (5) attempted to use titanous chloride as a reducing agent. Aihara and Soto (6) reported that ascorbic acid and certain other vitamins interfere with the assay. The interference of ascorbic acid has been confirmed in this laboratory and, in addition, low results have been obtained when large amounts of ferrous salts were present in the pharmaceutical preparation being assayed.

In an effort to improve this method of assay we were attracted to the work of Allfrey, *et al.* (7), who showed that the oxidation of folic acid with $KMnO_4$ in an acid medium yields a fluorescent compound useful as a basis for a fluorometric determination of the substance. In examining this reaction, we learned that permanganate oxidation in a buffered, mildly alkaline medium (K_2HPO_4) split the folic acid to the pteridine and aromatic amine moieties in a manner comparable to the zinc reduction procedure. Also, it was found that digestion of the preparation containing folic acid was accomplished better in this buffered medium rather than in the NH_4OH medium prescribed in the present U. S. P. method.

Another refinement is the extraction of the final colored product with isobutyl alcohol in those instances where extraneous colored materials are present which interfere with the color measurement.

We have found that a method incorporating these steps eliminates uncertainties resulting from the use of various forms of reductant and saves considerable assay time since folic acid is split by the permanganate oxidation in a shorter time than by the reduction procedures. Further, ascorbic acid and ferrous salts do not interfere.

We present here in detail the method we have found satisfactory and the experimental results that are the basis for its acceptability.

METHOD

Reagents.—Potassium Phosphate Solution (Dibasic), 3%—Dissolve 60 Gm. of reagent grade anhydrous K_2HPO_4 in H_2O to make 2,000 ml.

Potassium Permanganate, 4%—Dissolve 20 Gm. of reagent grade $KMnO_4$ in H_2O to make 500 ml.

Potassium Permanganate, 0.4%—Dilute 10 ml. of the 4% $KMnO_4$ solution with H_2O to make 100 ml. Prepare fresh solution for each assay.

Sodium Nitrite, 2%—Dissolve 2 Gm. of reagent grade $NaNO_2$ in H_2O to make 100 ml.

Hydrochloric Acid, 5 *N*—Dilute 44.5 ml. of reagent grade (35–37%) HCl with H_2O to make 100 ml.

Ammonium Sulfamate, 5%—Dissolve 5 Gm. of reagent grade $NH_4SO_3.NH_2$ in H_2O to make 100 ml.

N-(1-Naphthyl)-Ethylenediamine Dihydrochloride, 0.1%—Dissolve 100 mg. of reagent grade N-(1-Naphthyl)-ethylenediamine Dihydrochloride in

* Received November 8, 1958 from the Division of Nutrition, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.

† Present address: Division of Pharmacology, Food and Drug Administration.

H_2O to make 100 ml. Prepare fresh solution for each assay.

Sodium Chloride—Reagent grade NaCl.

Isobutyl Alcohol—Grade suitable for spectrophotometric and fluorometric use.

Folic Acid Standard Solutions.—*Stock Solution.*—Weigh accurately, in a closed system, U. S. P. Folic Acid Reference Standard, equivalent to 50–60 mg. of folic acid, that has been dried to constant weight and stored in the dark over P_2O_5 in a desiccator. Dissolve in about 50 ml. of 0.4% NH_4OH solution, and dilute with H_2O to make the folic acid concentration exactly 500 μg. per ml. Store under toluene in the dark at about 10°.

Standard Solution.—Dilute 2 ml. of the Folic Acid Stock Solution with the 3% K_2HPO_4 solution to make 100 ml. Each ml. contains 10 μg. of folic acid. Designate this as the Standard Solution. Prepare fresh Standard Solution for each assay.

Assay Solution.—Place a measured amount of the sample in a flask and add a volume of the 3% K_2HPO_4 solution equal in ml. to not less than 10 times the dry weight of the sample in Gm.; the resulting solution must contain not more than 0.1 mg. of folic acid per ml. If the sample is not readily soluble, comminute so that it may be evenly dispersed in the liquid.

Heat the mixture at 50–60°[1], agitating vigorously, and cool to room temperature. Allow any undissolved particles to settle. Filter or centrifuge if necessary. Dilute an aliquot of the clear solution with 3% K_2HPO_4 solution to a final measured volume that contains, per ml., about 5 μg. of folic acid. Designate this as the Assay Solution.

Assay Tubes.—To each of six or more tubes or reaction vessels, 40-ml. size, add 2.0 ml. of Assay Solution. Designate and prepare the tubes as follows: *Tubes A.*—To each of two or more of the tubes add 3 ml. of 3% K_2HPO_4 solution, and mix. *Tubes B.*—To each of two or more of the tubes add 1 ml. of the Standard Solution and 2 ml. of the 3% K_2HPO_4 solution, and mix. *Tubes C.*—To each of the remaining two or more tubes add 3 ml. of the 3% K_2HPO_4 solution, and mix.

To each tube designated A and B add, with mixing, 1 ml. of the 0.4% $KMnO_4$ solution and allow to stand two to three minutes. (Any appreciable change in the characteristic intense purple permanganate color during the two to three minute oxidation step denotes an excess of oxidizable material. Increase the quantity of $KMnO_4$ for assay solutions containing an excess of oxidizable material, but not more than about 1 ml. over that required to complete oxidation of foreign material.) To each tube designated C, add 1 ml. of H_2O and mix. (Whenever the volume of $KMnO_4$ solution is increased, add an equivalent volume of H_2O to each tube designated C so that all tubes are of the same volume.) To each tube designated A, B, and C, add 1 ml. of the 2% $NaNO_2$ solution; followed by 1 ml. of the 5 N HCl solution, and mix. Allow to stand two minutes; then to each tube add 1 ml. of the 5% $NH_4SO_3 \cdot NH_2$ solution, and mix. Swirl tubes vigorously until excess NO_2 is expelled (Excess of NO_2 will interfere with color development.) To each tube add 1 ml. of the 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution, mix, allow to stand ten minutes for maximal color development, and proceed as directed under (a) or (b).

(a) *For assay solutions that contain no extraneous color in an amount that interferes with final colorimetric measurements.*—Within the next fifteen minutes (but not more than twenty-five minutes after addition of N-(1-naphthyl)-ethylenediamine dihydrochloride, using water as the "blank," measure the absorbance of the solution from each tube as directed under *Determination.*

(b) *For assay solutions that contain extraneous color in an amount that interferes with final color measurements.*—To each tube add 1 Gm. of NaCl, and 10 ml. of isobutyl alcohol, and shake vigorously two to three minutes. Centrifuge tubes until 7–9 ml. of clear supernatant isobutyl alcohol solution can be obtained from each. Within twenty-five minutes after addition of N-(1-naphthyl)-ethylenediamine dihydrochloride, using isobutyl alcohol as the "blank," measure the absorbance of the isobutyl alcohol solution from each tube as directed under *Determination.*

Determination.—Measure the absorbance of the solution from each tube under A, B, and C at 550 mμ in a suitable photoelectric colorimeter set at 0 absorbance with the "blank".

Calculation.—Calculate on basis of aliquots taken as follows, using the absorbances of A, B, and C: Mg. of folic acid in sample = $[(A - C)/(B - A)] \times 0.005 \times$ dilution factor.

EXPERIMENTAL

In order to standardize and test the validity of certain critical steps in the method, in some of the experiments to be described, values resulting from known amounts of folic acid were compared with values resulting from equimolar amounts of p-aminobenzoic acid (PABA).[2] In these instances, the folic acid samples in 5-ml. volumes were treated with the same volumes and concentrations of reagents (except where noted) and in the same order as described under *Method.* Similarly, PABA was diazotized in a volume (8 ml.) equal to that in which the folic acid samples were diazotized. All spectrophotometric measurements were made in 10-ml. volumes.

Optimal Conditions for Splitting Folic Acid by Permanganate Oxidation.—Solutions containing 10 and 20 μg. of folic acid were oxidized for three minutes with 1 ml. of each of several concentrations (from 0.2% to 4.0%) of $KMnO_4$. In addition one set of aliquots of each concentration of folic acid was oxidized with *1 ml. of 0.4% $KMnO_4$ for six* minutes. Duplicate determinations were made in all cases. Following destruction of excess $KMnO_4$, diazotization of the folic acid split product and coupling with the Bratton-Marshall reagent, absorbance values of the solutions were compared with those resulting from equimolar amounts of PABA. When 1 ml. of $KMnO_4$ in concentrations up to 0.4% was the oxidant, even for periods as long as six minutes, absorbance readings of the final solutions did not differ significantly from those given by PABA solutions. With higher concentrations

[1] To avoid high blank readings, temperature during preparation of assay solution must not exceed 60°. At higher temperature diazotizable amines may be formed in the hydrolysis of organic material.

[2] The former U. S. P. Reference Standard for folic acid

of $KMnO_4$, however, absorbance readings of the treated folic acid solutions were significantly less than readings obtained from PABA solutions.

In order further to test the validity of the oxidation procedure, using 0.4% $KMnO_4$ and an oxidation time of three minutes, as previously described, absorbance values found were compared with those given by the same amounts of folic acid treated according to the zinc reduction procedure (1). The results, shown in Table I, show no significant differences in absorbance values obtained by the two procedures. Further, a comparison with absorbance values found for equimolar amounts of PABA also showed no significant differences and demonstrated a quantitative splitting of folic acid to PABA when using either method.

TABLE I.—ABSORBANCE READINGS RESULTING FROM FOLIC ACID SPLIT BY OXIDATION AND REDUCTION PROCEDURES AND FROM EQUIMOLAR AMOUNTS OF PABA

Sample No.	Absorbance[a] for 10 μg. Original Folic Acid, in 10 ml. of Final Solution: $KMnO_4$ Oxidation	Zinc Reduction	Absorbance[a] for Equivalent Amounts of PABA, in 10 ml. of Final Solution
1	0.175	0.170	0.175
2	0.180	0.175	0.171
3	0.170	0.175	0.170
4	0.175	0.175	0.180

[a] 1 cm. light path at 550 mμ.

Selection of Assay Medium.—In preliminary work on the method, experiments were performed to find a suitable medium for digestion of pharmaceutical preparations and for solution of the folic acid and, at the same time, provide a medium in which cleavage of the folic acid with $KMnO_4$ could take place quantitatively. Solutions of various concentrations of NH_4OH, $NaC_2H_3O_2$, $NaHCO_3$, and K_2HPO_4 were tried. It was not possible to obtain a quantitative recovery of folic acid when the oxidation took place in NH_4OH. With sodium acetate, the acetate ion interfered with the diazotization and coupling steps that followed. $NaHCO_3$ (5%) was satisfactory and gave about the same pH as a 3% K_2HPO_4, but the latter was finally selected because of its characteristic of precipitating ferrous ion.

Efficiency of Extracting the Chromogenic Compound with Isobutyl Alcohol.—With some pharmaceutical preparations, extraneous color was present in the final solutions which interfered with the color measurements. It was found that the chromogenic compound could be efficiently separated from foreign color by extracting it from a 10-ml. aqueous volume in the presence of 1 Gm. of NaCl, with 10 ml. of isobutyl alcohol.

Accordingly, absorbance readings of aqueous solutions of the colored compound, resulting from 10 and 20 μg. of folic acid, were compared with absorbance readings, at the same wavelength, obtained after extraction into isobutyl alcohol. The maximum absorbance for both solutions was at 550 mμ. It can be seen from Table II that the extraction took place with no diminution in the intensity of the color.

Effect of $FeSO_4$ and Ascorbic Acid on Folic Acid Assay, Using the K_2HPO_4 Digestion-$KMnO_4$ Oxidation Procedure.—Solutions containing known amounts of folic acid, and solutions of the same concentration of folic acid to which known amounts of $FeSO_4$ or ascorbic acid, and $FeSO_4$ and ascorbic acid were added, were assayed in duplicate as described under *Method*, using a new Folic Acid Reference Standard (see *Reagents*).

The solutions were as follows: Solution 1—1 mg. folic acid in 100 ml. 3% K_2HPO_4; 2—1 mg. folic acid in 100 ml. 3% K_2HPO_4 + 150 mg. ascorbic acid; 3—1 mg. folic acid in 100 ml. 3% K_2HPO_4 + 250 mg. $FeSO_4$; and 4—1 mg. folic acid in 100 ml. 3% K_2HPO_4 + 250 mg. $FeSO_4$ + 150 mg. ascorbic acid.

The amounts of $FeSO_4$ and ascorbic acid added approximated, or were greater than, amounts generally encountered in pharmaceutical preparations.

Comparison of folic acid values found for the four solutions showed no significant differences, indicating neither $FeSO_4$ nor ascorbic acid interfered in the assay.

TABLE II.—COMPARISON OF ABSORBANCE VALUES OF THE CHROMOGENIC COMPOUND IN AQUEOUS AND ISOBUTYL ALCOHOL SOLUTIONS

Solution	Absorbance[a] per 10 μg. Original Folic Acid, in 10 ml. of Solution	Absorbance[a] per 20 μg. Original Folic Acid, in 10 ml. of Solution
Aqueous (Before extraction)	0.175 0.170	0.350 0.360
Isobutyl Alcohol (After extraction)	0.165 0.170	0.340 0.355

[a] 1 cm. light path at 550 mμ.

In another experiment, to 1-ml. portions of four different liver extract preparations, each containing 5 mg. folic acid per ml. were added, in one instance, ascorbic acid and $FeSO_4$, and in another instance, ascorbic acid, $FeSO_4$, and an additional 5 mg. of folic acid. The solutions were assayed by the zinc reduction procedure (1) and by the K_2HPO_4 digestion-$KMnO_4$ oxidation procedure as described under *Method*, using the isobutyl alcohol extractions. Duplicate determinations were made in most cases.

The solutions were as follows: Solution *A*.—1 ml. of the liver extract; *B*.—1 ml. of the liver extract + 200 mg. ascorbic acid and 300 mg. $FeSO_4$; and *C*.—1 ml. of the liver extract + 200 mg. ascorbic acid, 300 mg. $FeSO_4$, and 5 mg. folic acid. This series of solutions were examined with each of four liver extract samples. Results obtained with the two procedures are shown in Table III.

It can be seen that, using the $KMnO_4$ oxidation procedure with K_2HPO_4 digestion, values found for Solutions *B* and *C* for the 4 samples were as expected when compared, respectively, with Solution *A*. Recoveries of added folic acid, calculated from values found for Solutions *A* and *C* of the samples, ranges from 96 to 100%. However, values obtained with the zinc reduction procedure with NH_4OH digestion were always low when ascorbic acid and $FeSO_4$ were present (Solutions *B* and *C*). In some instances absorbance readings of the controls were so high (for

TABLE III.—COMPARISON OF FOLIC ACID VALUES FOUND BY THE $KMnO_4$ OXIDATION AND BY THE ZINC REDUCTION PROCEDURES, IN THE PRESENCE AND ABSENCE OF ASCORBIC ACID AND $FeSO_4$

Sample	Average Folic Acid Found, mg — $KMnO_4$ Oxidation (Using K_2HPO_4 Digestion) Solutions A^a	Solutions B^a	Solutions C^a	Zinc Reduction (Using NH_4OH Digestion) Solutions A^a	Solutions B^a	Solutions C^a
1	6 2	6.1	11.1	6 2	[b]	[b]
2	4 8	4.8	9.9	4.9	[b]	[b]
3	6 4	6.3	11.1	6.1	0 9	3.4
4	4 4	4 3	9.2	4 1	1 1	2.7

[a] See text for composition of Solutions [b] Values found were negative and meaningless, see text

example with Solutions B and C of Samples 1 and 2) that when folic acid values were calculated, they were negative and meaningless Folic acid values for the unsupplemented Solutions A, as determined by the two methods studied, compared favorably, indicating that the liver extract solutions contained no interfering substances

Recovery of Folic Acid Added to Pharmaceutical Preparations.—Ten pharmaceutical preparations were assayed for folic acid using the K_2HPO_4 digestion-$KMnO_4$ oxidation procedure as described under *Method* Concurrently, the same preparations to which were added known amounts (5 or 10 mg) of folic acid were assayed Values for the two sets were compared respectively, and recovery of folic acid in each case was calculated. Recoveries ranged from 94 to 102%, with an average recovery of 98 7%

Analyses of Pharmaceutical Preparations for Folic Acid Content by $KMnO_4$ Oxidation and Zinc Reduction Procedures.—In order to compare the two procedures, several preparations consisting of injectable solutions, capsules, and tablets containing $FeSO_4$, liver fractions, yeast, vitamins, and other constituents in various amounts and combinations, were assayed in duplicate and the folic acid values compared Samples 1 to 5, according to label declarations, contained ferrous compounds and ascorbic acid, while samples 6 to 10 did not. As can be seen from Table IV, good agreement between the two methods was obtained only when these interfering substances were absent

DISCUSSION

These experiments indicate that the permanganate oxidation procedure is applicable to a variety of pharmaceutical preparations

The critical step in the proposed procedure is in the splitting of the folic acid Although high concentrations of $KMnO_4$ or prolonged periods of oxidation may result in oxidation of folic acid to compounds other than the pteridine and PABA or, what is more probable, partial destruction of these primary split products, there is a satisfactory range of concentration and time that permits safe use. From reports in the literature (3, 4) and experience in this laboratory, cleavage of the folic acid molecule is also a critical step in the reduction procedures. In the method proposed, effects of excessive $KMnO_4$ oxidation are offset since the reference standard and assay solutions are exposed to the same oxidation influences A similar approach used in the assay of riboflavin (1) has proved useful

The interference of ferrous salts and ascorbic acid in the present U S P method and the absence of their interference in the proposed oxidation procedure may be explained on the basis that these substances themselves, as present in pharmaceuticals, act as reducing agents

In the reduction procedure ferrous ion may not be completely precipitated by NH_4OH so that it, or the ascorbic acid still present, may cause some reduction in the initial digestion step Another possibility is that these reductants might exert an auxiliary reducing effect later when the reduction is supposedly attributed solely to zinc

With the proposed procedure, either the ferrous ion may be more completely precipitated, or the lower pH of the K_2HPO_4 solution may inhibit reduction by it or by ascorbic acid. Furthermore, since the cleavage of folic acid is brought about by $KMnO_4$ oxidation, this oxidation may, at the same time, nullify reducing effects of the ferrous ion or ascorbic acid present.

These concepts are in agreement with our findings since, in all cases where ferrous compounds or ascorbic acid were present, low results, indicating excessive folic acid reduction, were encountered with the reduction procedure, but not with the oxidation method.

TABLE IV.—ANALYSES OF PHARMACEUTICAL PREPARATIONS FOR FOLIC ACID BY $KMnO_4$ OXIDATION AND ZINC REDUCTION METHODS

Sample No	Folic Acid Present (Label Declaration)	Folic Acid Found, mg — $KMnO_4$ Oxidation Procedure Analysis No 1	Analysis No 2	Zinc Reduction Procedure Analysis No 1	Analysis No 2
1[a]	3 mg /tablet	3 8	4 1	2.2	2 6
2[a]	3 mg /tablet	2 8	3 1	1 4	1 5
3[a]	5 mg./tablet	6 1	6.3	0 9	1.0
4[a]	5 mg /ml	4 3	4 8	1 1	1 2
5[a]	1 mg./capsule	1 1	1 3	0.4	0 4
6	10 mg./ml	11 3	11.8	11 5	11 9
7	1 mg /tablet	1 0	0.9	0.8	0.8
8	1 mg./ml.	1.3	1.4	1 1	1.3
9	5 mg /3 tablet	3 8	3 5	3.7	3.3
10	0 5 mg /capsule	0 6	0 6	0 6	0.7

[a] According to label declaration, these samples contained ferrous compounds and ascorbic acid.

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A Colorimetric Assay for Unoxidized Phenothiazine Derivatives: A New Complex Salt*

By JAMES A. RYAN

The lauryl sulfate salts of palladium phenothiazine derivative complexes provide a colorimetric means of following oxidative decomposition in such pharmaceutically important compounds as promazine, promethazine, and chlorpromazine. This rapid and highly specific method is well suited to routine assay of the intact drugs. The need for initial separation is eliminated in many cases.

RECENT EMPHASIS upon phenothiazine derivatives because of their importance as tranquilizing and antihistaminic agents has again focused the analytical chemist's attention on this important field. Several types of decomposition appear to take place in these compounds. One type is a mild oxidation at the sulfur atom leading to the 5-oxide or sulfoxide and eventually to the 5-dioxide or sulfone (1). Quinoid type oxidation products of phenothiazine are discussed by Granick, Michaelis, and Schubert (2–4). However, in aged or badly decomposed pharmaceutical preparations from accelerated stability studies, the extent of decomposition, or the amount of each type of decomposition product present has posed difficult problems to the analytical chemist.

The literature is replete with methods employing oxidizing agents as reagents for assaying the parent compound and its derivatives. Typical of these methods are the color reactions of Eddy and DeEds (5), Dubost and Pascal (6), and Newhoff and Auterhoff (7). The ultraviolet method of Scott (8) has been applied to measure oxidation by evaluation of the absorbance ratio of two maxima. The pure number obtained is without correlation to amount of intact material remaining in complex oxidation mixtures. The assay values obtained increase with increasing decomposition.

Failure of these methods to provide an adequate measure of intact material during stability studies on such compounds as promazine and promethazine led to an investigation of complex formation via the oxidation-labile sulfur atom as a colorimetric approach to the solution of this problem.

EXPERIMENTAL

The chlorides of gold and the group VIII transition metals were combined in dilute unbuffered solution with certain phenothiazine derivatives. The palladium complex was then selected for further investigation. Overholser and Yoe (9) used phenothiazine to determine palladium ion. These authors as well as Marcali (10) mention difficulty experienced due to the low solubility of the complex. This complex has essentially the required qualification, i. e., inability to react with the oxidized sulfur atom. Carkhuff and Boyd (11) reported a titration procedure using sodium lauryl sulfate as a titrant for quaternary and certain tertiary amines. Inasmuch as compounds such as promazine, promethazine. and chlorpromazine have the necessary molecular qualifications for forming slightly dissociated salts with the lauryl sulfate ion on the acid side, it was decided to try the lauryl sulfate ion with the phenothiazine derivative palladium complex in an effort to enhance the color formation. This resulted in the formation of a new complex salt whose visible spectrum is reproduced in Fig. I. Note that when compared to the chloride salt the result is an approximate twofold increase in the sensitivity for promethazine accompanied by a bathochromic shift, see Equation I. Color development was poor if the lauryl sulfate ion was added prior to addition of the sample to the solution of palladium chloride, see Equation II. This suggests the complex formation involves initial formation of the palladium-phenothiazine derivative complex followed by replacement of the ionic chlorides associated with the side chain nitrogen as well as those associated with the palladium atom, palladium lauryl sulfate failing to react well with the phenothiazine derivative. A wavelength shift is also noted when the lauryl sulfate ion is added to the palladium phenothiazine complex again substantiating replacement of the ionic chlorides associated with the palladium atom, see Equation III.

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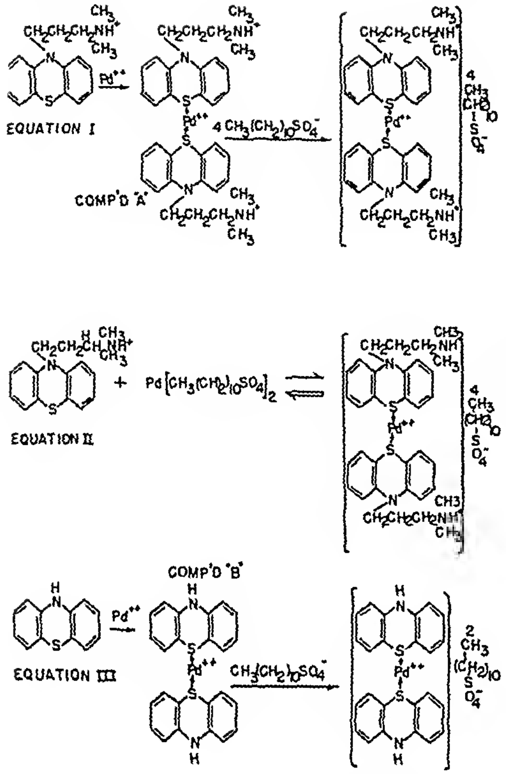

The equations are postulated for the mechanisms involved and are supported by all experimental evidence. A compound *B* above is in agreement with the elemental analysis as determined by Overholser and Yoe (9). Compound *A* was prepared (12) and complete elemental analysis confirmed a 2:1 molar ratio of promazine hydrochloride to palladium chloride. After the addition of four equivalent weights of magnesium lauryl sulfate to a solution of *A* no further change in the spectrum was noted. This experiment was carried out in a large volume of a dilute solution of *A* and the changes in the spectrum produced were followed spectrophotometrically. To support coordination involving the unoxidized sulfur atom with the palladium ion the latter was combined in dilute unbuffered solutions with the following compounds: acridine, phenoxazine, phenazine, promazine sulfoxide, and phenothiazine sulfone.

With the exception of acridine which yielded a strong greenish fluorescence and a yellow solution to transmitted light, the above compounds failed to show any visible signs of reaction.

To illustrate the applicability of this reaction to the assay of an intact phenothiazine derivative in the presence of its oxidized product, as well as a confirmation of the adherence of the assay procedure subsequently presented to the Beer-Lambert laws, a series of standard solutions of promazine hydrochloride and its sulfoxide in varying proportions of each was subjected to the assay method presented below. Results are indicated in Fig. 2.

Spectra of the Complexes

Figure 3 shows the visible spectra of promazine, chlorpromazine, and promethazine as their palladium lauryl sulfate complex salts plotted con-

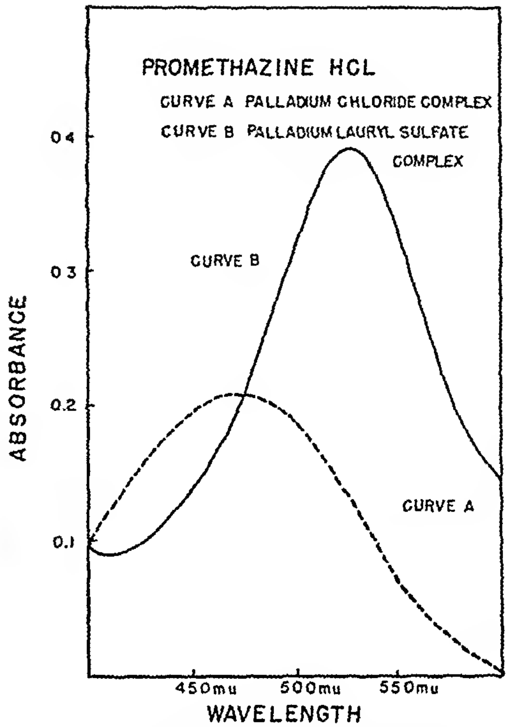

Fig. 1.—Spectra of the chloride and lauryl sulfate salts of the palladium promethazine complex.

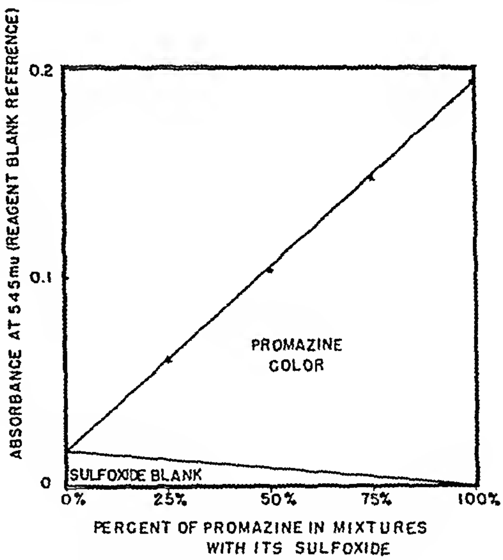

Fig. 2.—Beer's law adherence of the method in the presence of the sulfoxide.

gruently. The absorption maxima are 545 mμ, 545 mμ, and 525 mμ, respectively.

Compound *A* when compared in the infrared to its starting material revealed a number of significant spectral differences which are indicated in Fig. 4.

Conventional potassium bromide pellet technique was used in the preparation of the infrared spectra, Fig. 4. Concentrations used were 0.3 and 0.4% in potassium bromide, employing a 1-mm. path. The

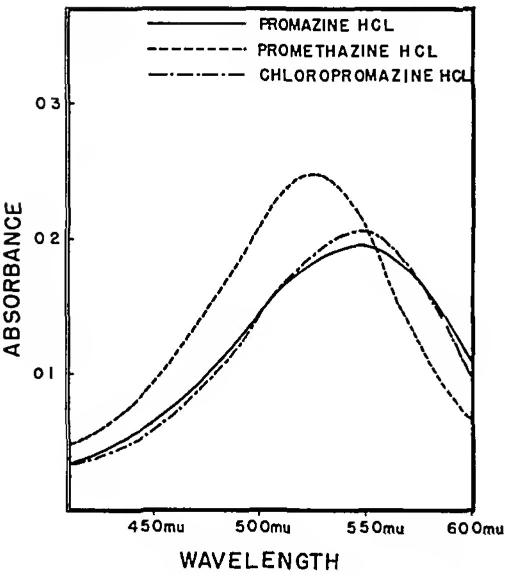

Fig 3—Spectra of the palladium lauryl sulfate complex salts of promazine, promethazine, and chlorpromazine

instrument used was a Perkin Elmer Model 21 The visible spectra are reproductions of graphs as recorded on a Beckman DK-2 instrument with linear wavelength attachment Single measurements were made on a Beckman DU spectrophotometer

Analytical Method

Reagents.—*Stock Palladium Chloride Solution*—Weigh 500 mg of palladous chloride into a 250-ml. beaker, add 5 ml of concentrated hydrochloric acid and warm the mixture on the steam bath Add 200 ml of hot water in small increments with continued heating until solution is complete Transfer the solution to a 500-ml volumetric flask and fill to the mark at room temperature with water

Buffered Palladium Chloride Solution—Transfer 25 0 ml of stock palladium chloride solution to a 500-ml volumetric flask, add 50 ml of normal sodium-acetate solution, 48 ml of normal hydrochloric acid, dilute to volume, and mix well

Magnesium Lauryl Sulfate Solution—A 0 1% aqueous solution The magnesium salt is preferred because the solutions have less tendency to haze and precipitate Dioctyl sodium sulfosuccinate appears to work equally well, but its salts have not been investigated in relation to this work

Assay.—The colored test solution is prepared as follows: Transfer 3 0 ml of the buffered palladium chloride solution to a 25-ml Erlenmeyer flask Add 2 00 ml of an aqueous standard solution representing 100 to 125 μg of the pure phenothiazine derivative for standards, or an aqueous sample solution diluted to the same level Mix well Add 2 0 ml of the magnesium lauryl sulfate solution and mix well Sample and standard solutions are prepared in duplicate The absorbances of these solutions are determined on a suitable spectrophotometer or colorimeter, the absorption maximum of the particular complex being utilized The instrument is set at zero absorbance using a reference solution composed of 3 0 ml. of buffered palladium chloride solution, 2 0 ml of water, and 2 0 ml. of the magnesium lauryl sulfate solution The concentration of the sample is then calculated If the sample is highly colored it may be necessary to employ a sample color blank This is prepared by omitting the buffered palladium chloride solution and substituting water in the same volume Absorbances of all solutions are then determined using water as a reference liquid and appropriate corrections applied

Preparation of Sample.—In most cases the only operation necessary for injections is a direct dilution to the concentration level mentioned above In the case of tablets or capsules the following technique is usually successful. Crush a number of tablets or empty a number of capsules representative of the batch under test and grind until a homogeneous powder is obtained Transfer an amount of the powder equivalent to approximately 10 mg of the derivative to a glass-stoppered flask containing exactly 200 ml of distilled water Stopper and shake until complete solution of the active ingredient is effected. Filter through a Whatman No 42 paper, rejecting the first 150 ml of the filtrate Remove a 2 00-ml aliquot of the subsequent filtrate for the determination

Precision and Interferences

Based upon thirteen standard solutions prepared in the manner indicated above, the average deviation of a single measurement proved to be 0 001 absorbance unit No deviation from the arithmetical mean greater than ±0 003 absorbance unit was encountered in this series

The following typical substances commonly found in tablets and capsules did not interfere with color development at the levels indicated after aqueous solution of 5 0 mg of promethazine hydrochloride in their presence

Acetophenetidin	161 mg.
Acetylsalicylic acid	226 mg.
Caffeine	32 mg.
Codeine sulfate	16 mg.
Meprobamate	200 mg.
Corn starch	60 mg.
Lactose	500 mg
Magnesium stearate	10 mg
Methocel 400	15 mg
Sterotex	2 mg

Phenol and sulfur-containing antioxidants were found not to interfere in the usual concentrations at which they appear in injections. If substances which react with palladium chloride or are incompatible with the lauryl sulfate ion are present, caution should be exercised in applying the method High concentrations of acids, bases, salts, and organic solvents should be avoided.

If interference of any type is suspected on a given product, the test of interference should be the congruency of the visible spectrum of a complete control and a standard.

Examples of Application of the Assay to Accelerated Stability Studies

The data presented below on two selected products shows how the palladium lauryl sulfate method may be used to evaluate product stability. A choice between two different closures can be made with con-

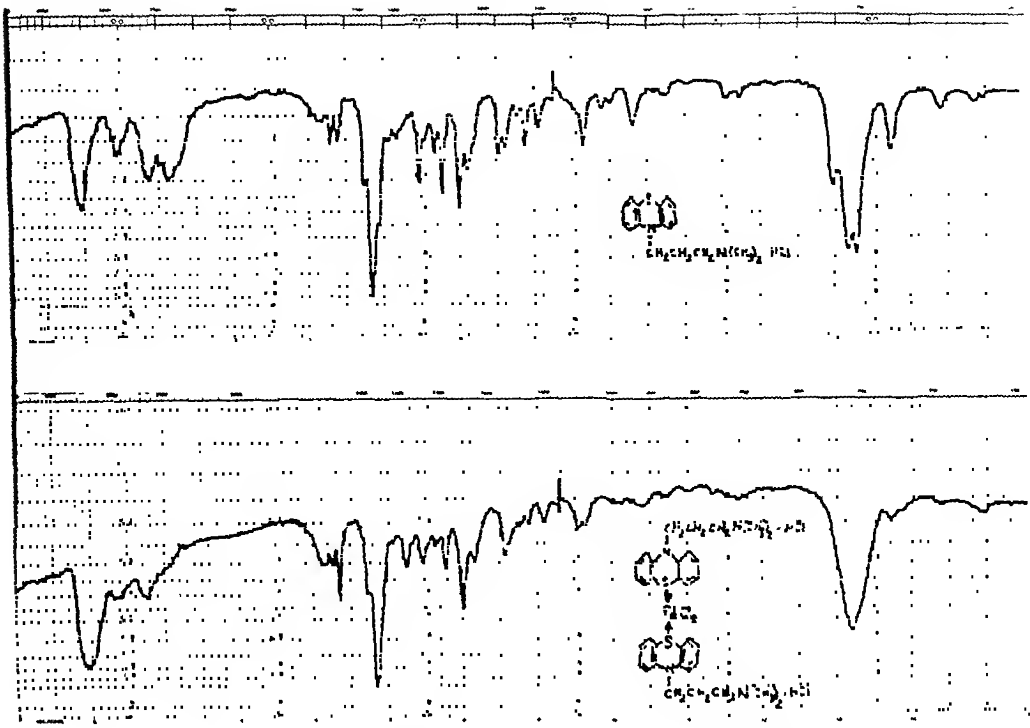

Fig. 4.—Infrared spectra of promazine hydrochloride as compared to that of its palladium complex.

fidence. The previously mentioned ultraviolet method of Scott (8) was employed below.

Example A.—Promethazine hydrochloride injection 25 mg./ml. containing a narcotic present at the same concentration level.

	Ultraviolet Method, mg./cc.	Ratio $\frac{A_{249\ m\mu}}{A_{298\ m\mu}}$	Palladium Lauryl Sulfate Method, mg./cc.
Initial	24.4	8.35	...
Six months storage under U. V. light	26.6	7.13	20.3
Six months, 45°	24.1	8.44	23.0

Example B.—Promethazine hydrochloride injection 25 mg./ml.

Initial Assay, U. V. method—26.0 mg./ml. or 104% of claim.

Storage assays by the palladium lauryl sulfate method.

Closure No. 1	Closure No. 2
25.5 mg./cc. or 102% of claim	15.3 mg./cc. or 61.3% of claim

The storage conditions of example *B* study were 45° for two years and room temperature for seven months.

SUMMARY

A method for the analysis of the more common phenothiazine medicinals has been presented. This method based on the lauryl sulfate salt of the specific palladium phenothiazine derivative complexes provides a linear measure of oxidative decomposition at the sulfur atom of these compounds.

Routine evaluation of raw material condition and product stability under accelerated storage is simplified. Choice of containers and closures is expedited.

Spectra, postulated structure, and reactions of these complexes are discussed.

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Enteric Coatings II*

Starch (and Amylose) Acetate Phthalates

By JOHN G. WAGNER, THOMAS W. BRIGNALL, and STUART LONG

Eight new whole starch (and amylose) acetate phthalates differing in acetyl and phthalyl contents were prepared by acetylation of a reactive form of starch or amylose with acetic anhydride in the presence of formamide followed by phthalylation of the partial acetates with phthalic anhydride in the presence of pyridine. Solubilities of the compounds indicated they would be useful substances for enteric coating. Tablets were enteric coated with one of the starch acetate phthalates and a commercial cellulose acetate phthalate. *In vitro*, at pH 6.9, ox bile extract but not pancreatin increased the rate of disintegration of the enteric coated tablets; hence, under these conditions, ionization of free carboxyl groups and not the action of enzymes in pancreatin is mainly responsible for the disintegration of these enteric coated tablets.

During the past several decades various substances and combinations of substances have been evaluated for enteric properties and a variety of artificial gastric and intestinal fluids and *in vitro* testing apparatus have been studied (1).

This is the second in the series[1] of publications which will describe the preparation and properties of some new enteric substances, discuss the factors involved in the disintegration of enteric coatings *in vitro* and *in vivo;* show that certain mathematical relationships exist which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another, and correlate *in vivo* with *in vitro* disintegration times.

An enteric polymer may dissolve because of ionization of an acidic function in its molecule. For example, a cellulose acetate phthalate was reported (2) to dissolve at a pH as low as 5 7. This would be expected to cause disintegration of the enteric coating in which such a polymer was contained. However, Bauer and Masucci (3) claimed that "The disintegration of enteric coatings like cellulose acetate phthalate in the intestinal contents, which are on the acid side, is the result of the hydrolytic action of intestinal esterases." Enzymatic attack on susceptible linkages of an enteric polymer would be of value from an enteric coating viewpoint only if such an attack occurred at a faster rate than the solution of the polymer due to ionization. Resolution of the relative importance of these two possible factors would aid the search for enteric substances with improved properties.

Model enteric substances have been studied by the present authors. These substances include commercial cellulose acetate phthalate, new starch (and amylose) acetate phthalates, and two synthetic polymers which have not formerly been used in enteric coatings Cellulose acetate phthalates and starch (and amylose) acetate phthalates have phthalate and acetate ester groups which are presumably susceptible to enzymatic attack. Startch (and amylose) acetate phthalates have (1⟶4)-0-α-D-glucosidic linkages presumably susceptible to attack by intestinal enzymes, whereas cellulose acetate phthalates have (1⟶4)-0-β-D-glucosidic linkages in the cellulose chains which are not attacked by intestinal enzymes. The synthesis of starch (and amylose) acetate phthalates and preliminary investigation of the relative importance of enzymatic attack and ionization in promoting the disintegration of enteric coatings containing a cellulose acetate phthalate or a starch acetate phthalate are given below.

Starch consists (4, 5, 6) of a mixture of a linear polymer, amylose, and a branched polymer, amylopectin. Amylose consists of anhydrous glucose units linked by (1⟶4)-0-α-D-glucosidic bonds Amylopectin is composed of anhydrous glucose units linked by the same type of glucosidic bonds but with branches in the 6-position. The preparation of neither whole starch acetate phthalates nor amylose acetate phthalates has been described before.

EXPERIMENTAL

Materials.—Amylose paste, 15–20% butanol-covered (The G Frederick Smith Chemical Co), buffalo corn starch (Corn Products Refining Co), formamide 99% and phthalic anhydride #2619 (Matheson Coleman & Bell, Inc), acetic anhydride A. R. and pyridine A R (Mallinckrodt Chemical Works),

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1 The first paper in this series is reference (14)

pancreatin triple strength U. S. P. (The Wilson Laboratories); and cellulose acetate phthalate type S1 (Eastman Organic Chemicals), *Anal.*—phthalyl 30.85%; acetyl 20.91%; free phthalic acid 1.75%.

SYNTHETIC METHODS

Preparation of Air-Dried Reactive Amylose.—The procedure described by other investigators (6, 7) was used. The contents of two one-ounce bottles of "15–20% amylose paste, butanol-covered" yielded 8.95 Gm. of 60-mesh air-dried reactive amylose.

Preparation of "Disintegrated Starch Granules". (*a*) *On a Laboratory Scale.*—The method of Wolff, Olds, and Hilbert (8) was utilized. Thirty-five grams of corn starch yielded 28 Gm. of "disintegrated starch granules" all of which passed through a 60-mesh screen and which contained 5 5% moisture.

(*b*) *On a Pilot Laboratory Scale.*—One kilogram of corn starch was slurried with 1,425 ml. of deionized water at room temperature and the slurry added gradually with vigorous stirring to 12,825 ml. of boiling distilled water. The paste was stirred vigorously and held at 90° for ten minutes. The temperature was raised to 100° and the paste run through a Martin-Gaulin colloid mill with a 0.002-inch setting. The slurry was cooled to 50–55° and then 14,250 ml. of denatured alcohol[2] was added slowly with vigorous stirring in such a manner as to avoid the formation of lumps of precipitated starch. The resulting suspension was run through a Fitzpatrick pulverizing machine with a 100-mesh screen. When the suspended particles had settled, the supernatant liquid was decanted; the same volume of fresh denatured alcohol was added and the suspension stirred vigorously for ten minutes. The decantation was repeated after the suspension had settled. The washing procedure was repeated twice using one-half the previous volume of denatured alcohol. The final product was isolated by filtration and dried in a vacuum oven at 80° for fifteen to eighteen hours. Yields of 650 to 725 Gm. of "disintegrated starch granules" which passed through a 60-mesh sieve and which contained about 5% moisture were obtained. Material failing to pass a 60-mesh sieve was discarded.

Acetylation.—The proportion of reagents reported by Potter and Hassid (9) was used. Acetylation in formamide was by an adaptation of the method of Jeanes and Jones (7). The modifications consisted of precooling both the formamide and pyridine to 10° ± 3° and maintaining the same temperature during the addition of the pyridine to the formamide-starch suspension; similarly the temperature was held at 10° ± 3° during addition of the acetic anhydride in increments over a fifteen-minute period (except in preparation of S. A. P. II; see Table 1). After all the acetic anhydride had been added, cooling was discontinued and the mixture stirred while the temperature was allowed to rise gradually to between 22 to 28°. A further modification consisted of stopping the reactions after the variable periods of time by pouring the reaction mixture into a mixture of ice and water. Table I gives the time of acetylation from the start of the addition of the acetic anhydride to the ice treatment for each product. In each case the resulting white, fibrous precipitate was recovered by filtration, washed well by resuspension four times in deionized water, dried in a vacuum oven at 80° for fifteen hours, then ground and passed through a 20-mesh sieve. The 20-mesh powder was dried to constant weight in a vacuum oven at 80°. The acetyl contents and yields of the various products are shown in Table I.

During the acetylation of a Kg. of "disintegrated starch granules" (S. A. II, see Table I), difficulty was experienced in keeping the temperature low during the addition of the acetic anhydride in ten two-liter portions. The reaction is exothermic and in spite of the circulating brine system, the temperature rose to 22°. Additions were discontinued until the temperature was again 10°. One hour was required to complete the addition while maintaining the temperature at 10° ± 2°. The brine system was removed and the temperature allowed to rise to 25°. Stirring was continued for three hours when a clear syrupy solution was obtained. The isolation was effected as described above. The resulting starch acetate analyzed 38.4% acetyl. Sixty grams of this was phthalylated to yield S. A. P. II but the remainder of the starch acetate was partially saponified to yield starch acetate (III) of lower acetyl content.

The partially acetylated starch (S. A. II) was partially saponified using the following proportions of reagents: starch acetate 14.5 Gm., acetone 207 ml., sodium hydroxide 0.5 Gm., and deionized water 1.0 ml. The starch acetate was dissolved in the acetone at reflux temperature. The sodium hydroxide was dissolved in the water and the solution added to the acetone solution of starch acetate. Heating and stirring was continued for two hours, then the reaction mixture was allowed to cool overnight. The reaction mixture was poured slowly into water and stirred for four hours. The product was isolated by centrifugation, washed well with water until neutral, and dried in a vacuum oven to constant weight at 50°. The product (S. A. III) analyzed 34.0% acetyl.

Phthalylation.—Phthalylation of the partially acetylated starch (or amylose) acetates was effected by phthalic anhydride in the presence of pyridine. The proportion of reagents reported by Malm and Fordyce (10) for the phthalylation of cellulose acetate was used. A mixture of 1 part by weight of the starch (or amylose) acetate, 4 parts of pyridine, and 2 parts of phthalic anhydride was heated to 100° with stirring and the temperature maintained for the times indicated in Table 1. The reaction mixture was usually in the form of a viscous gel; it was diluted with acetone and poured into a dilute solution of hydrochloric acid containing ice. The mixture was stirred until the gel had been converted to a white or tan, fibrous solid which was recovered by filtration. The fibrous solid was washed four times by resuspension in cold water, then five times in boiling water. The product was dried in a vacuum oven at 80° for three hours, cooled, ground, and passed through a 60-mesh sieve. The powder was dried to constant weight in a vacuum oven at 80°. A summary of the products is shown in Table I.

[2] Alcohol 3A which is prepared by mixing 100 parts, by volume, ethyl alcohol and 5 parts, by volume, commercially pure methyl alcohol

TABLE I —SUMMARY OF DATA ON STARCH (AND AMYLOSE) ACETATE PHTHALATES

	Reaction Conditions		Analytical Values A A P[c] or S A P[d]						Calculated Av No Groups/Anhydro-Glucose Residue		Yield, %	
Product	Acetylation	Phthalylation	A A[a] or S A[b] Acetyl, %	Acetyl[e] %	Phthalyl,[e] %	Free Phthalic Acid, %	Moisture, %	Calculated Carboxyl (−COOH), %	Acetyl	Phthalyl	Acetylation[f]	Phthalylation[e]
A A P. I.	5 hrs 10–26°	8 hrs, 100 ± 5°, r t overnight	39 9 39 4	32 8	13 4	8 4		4 1	2 22	0 28	.	
S A P II	4 hrs[j] 8–25°	18 hrs, 85–90°	38 4	34 7	17 4	2 25	1.34	5 3	2 67	0 39	90 0	98 9[h]
S A P III	[k]	18 hrs, 100 ± 5°	34 0	31 0 30 9	19 7 19 5	3 15 2 95	1 65 1 78	5 9	2 32	0 42	85 8[g,i]	85 2[g]
S A P IV	45 min, 7–22°	8 hrs, 100 ± 5°, r t overnight	33 3	25 3	22 2	11 7	2 0	6 7	1 86	0 46	75 4	94 1
A A P V	45 min, 10–21°	8 5 hrs, 100 ± 5°, r t overnight	31 5	22 0	25 9	9 0		7 8	1 57	0 53	59 4	82 5
S A P VI	40 min, 9 5–27°	20 hrs, 100 ± 5°		18 8	30 5	4 7	.	9 2	1 38	0 65	75 3	90 5
S A P VII	45 min, 7–22°	12 hrs, 100 ± 5°		16 2 15 6	30 8 31 1	2 46 2 16	4 62	9 4	1 11	0 63	97.4	63 7
S A P VIII	45 min, 7–22°	12 hrs, 100 ± 5°		23 7	32 7	3 3		9 9	2 01	0 80	84 8	75 7

[a] A A amylose acetate [b] S A, starch acetate [c] A A P, amylose acetate phthalate [d] S A P, starch acetate phthalate [e] On phthalic acid-free moisture free basis [f] Based on moisture free starch [g] Kilogram batch [h] Starting material was 60 Gm of S A II [i] Overall yield inclusive of partial saponification of S A II to yield S A III [j] Addition of acetic anhydride required one hour [k] S A III obtained by partial saponification of S A II

Analytical Methods.—(*a*) *Moisture*—About 2 Gm of starch (or amylose) acetate phthalate was weighed to the nearest mg in a dry, tared, glass-stoppered weighing bottle and dried for at least two hours in an oven at 105–110° The sample was cooled and weighed

(*b*) *Acetyl in Starch (or Amylose) Acetates*—The pyridine method of Murray, Staud, and Gray (11) was used

(*c*) *Free Phthalic Acid*—The ether extraction and titration method of Malm, Genung, and Kuchmy (12) was used

(*d*) *Phthalyl*—The titration method of Malm, Genung, and Kuchmy (12) was used The ether extracted material from the above determination was freed from ether in a desiccator and dried for two hours at 100–110° The solvent was a mixture of 1·1, by volume, pyridine and acetone Blanks were run on the solvent

(*e*) *Acetyl in Starch (or Amylose) Acetate Phthalates*—The apparent acetyl (total alkali consumption calculated to acetyl, equivalent weight 43) was determined by the saponification-in-solution method of Malm, Genung, Williams, and Pile (13) using a solvent mixture of 1 1, by volume, pyridine and acetone Analyses were performed on phthalic acid free samples as above The actual acetyl value was calculated by means of the equation·

$$\% \text{ acetyl} = \% \text{ apparent} - \left(\% \text{ phthalyl} \times \frac{86}{149}\right).$$

(*f*) *Carboxyl*—Calculated from · % free carboxyl = 45/149 × % phthalyl

SOLUBILITIES

All starch (and amylose) acetate phthalates prepared were readily soluble in 5% aqueous sodium hydroxide, 10% aqueous ammonia, acetone, methyl ethyl ketone, and mixtures of acetone or methyl ethyl ketone and alcohol, although alcohol itself was a nonsolvent At least 10% w/v solutions in the organic solvents could be prepared

All starch (and amylose) acetate phthalates prepared were soluble in 5% aqueous sodium bicarbonate with the exception of A A P I which had the lowest per cent carboxyl of the group. Similarly all products except A A P I were readily soluble in the artificial intestinal fluids, pH 6 9 (see Table III) All products were completely insoluble in simulated gastric fluid U S P XV

The solubility of some of the products in phosphate buffers was determined Ten milligrams of compound and 50 ml of buffer were placed in a stoppered bottle The bottles were attached to a wheel which rotated six revolutions per minute in a constant temperature bath held at 40° The bottles were examined after one hour and after four hours Phosphate buffers differing in pH by 0 05 pH unit were tried for each compound until that compound was just completely soluble after one hour and after four hours Table II lists the per cent carboxyl in

the compound and the minimum pH of the buffer in which it was soluble after four hours. In general, complete solution after one hour required a buffer from 0.05 to 0.20 pH unit higher than that given for four hours. The 3 products with 6.7, 7.8, and 9.2% free carboxyl content dissolved at a lower pH than the cellulose acetate phthalate with 35.2% phthalyl and 10.6% carboxyl reported by Malm, Emerson, and Hiatt (2).

TABLE II.—RELATIONSHIP BETWEEN THE PER CENT CARBOXYL IN THE STARCH (OR AMYLOSE) ACETATE PHTHALATE AND THE pH AT WHICH IT DISSOLVED

Per Cent Carboxyl	Minimum pH of Phosphate Buffer[a]
5.3	6.30
5.9	6.00
6.7	5.60
7.8	5.55
9.2	5.55

[a] Minimum pH of phosphate buffer at which 10 mg. of polymer just completely dissolved in 50 ml. of buffer at 40° after four hours.

COATING OF TABLETS

Compressed tablets containing 3 gr. of barium sulfate U. S. P. and lactose as diluent were prepared from a starch-syrup granulation. One lot was subcoated with a gelatin-syrup solution using a mixture of precipitated calcium carbonate, talc, and acacia powder as dusting powder. This lot was enteric coated with cellulose acetate phthalate by applying to the tablets a 10% w/v solution of the polymer in a mixture of acetone and alcohol. This lot of enteric coated tablets were those designated lot III in Table III of the previous publication (14). The other lot of compressed tablets was subcoated with a sodium carboxymethylcellulose-sugar-water solution (15) using the same dusting powder. This lot was enteric coated with starch acetate phthalate by applying to the tablets a 10% w/v solution of the polymer in a mixture of methyl ethyl ketone and alcohol. This lot of enteric coated tablets were those designated lot IV in Table III of the previous publication (14). U. S. P. supreme talc was used as dusting powder during the enteric coating of both lots of tablets.

The average weights of enteric polymer and dusting powder applied per tablet and the average volumes, weights, and thicknesses of the enteric coatings with their standard errors are given in the previous publication (14).

DISINTEGRATION TESTS

Artificial Gastric Juice, pH 1.2 and Water.—The *in vitro* resistance of the enteric coatings to simulated gastric fluid, U. S. P. XV and to water was reported in the previous publication (14).

Artificial Intestinal Fluids, pH 6.9.—The buffers shown in Table III were utilized.

The disintegration apparatus was that described in the U. S. P. (16). The temperature of the buffers were maintained at 37° ± 2°. Each run included six tablets in one basket and two runs were carried out on each lot in each buffer. The runs and samples were completely randomized. The end point for each tablet was taken when the tablet was completely disintegrated and 99 to 100% of the entire disintegrated tablet and coating had passed through the screen of the disintegration apparatus. The results of the disintegration tests in the four buffers are shown in Table IV. It was found that when the enteric coated tablets were run two hours in simulated gastric fluid U. S. P. XV then transferred to buffer A that the average values of disintegration time in buffer A were approximately the same as those in Table IV under buffer A where the enteric coated tablets were put directly into buffer A.

Comparison of Spread and Errors.—The average disintegration times of the enteric coated tablets in buffers A, B, C, and D were 45.0, 44.5, 55.0, and 55.0 minutes, respectively. The average disintegration times of the enteric coatings (obtained by subtraction of the average disintegration time of the subcoated tablets from the average disintegration time of the enteric coated tablets) in buffers A, B, C, and D were 38.8, 38.3, 47.8, and 46.7 minutes, respectively. Numerically the average disintegration time of the enteric coated tablets is 23% greater in buffers C and D which contain no ox bile extract than in buffers A and B which contain ox bile. Numerically the average disintegration time of the enteric coated tablets is about 0.5% higher in buffers A and C which contain pancreatin than in buffers B and D which do not contain pancreatin.

Barlett's test (17) was used to test the homogeneity of the variance of disintegration time in the four different artifical intestinal fluids. The test indicated some lack of homogeneity. However, despite the high χ^2 value an analysis of variance seems justified (18, 19). Based on a large number of similar disintegration tests run in this laboratory there is little evidence of a relationship between standard deviation and average disintegration time although such a relation is usually to be expected with time measurements. Occasional erratic differences in variances appears to be due mainly to nonuniformity in the coatings arising from the coating process. Some improvement in homogeneity was obtained by using log time; however, χ^2 was still significant.

Analysis of Variance.—Using the "time (T)" values directly, an analysis of variance of the data presented for the enteric coated tablets, lots III and IV in Table IV was made. The results of the analysis are shown in Table V.

The conclusions which may be drawn from the analysis of variance are as follows: (*a*) The pooled disintegration times of the tablets enteric coated with cellulose acetate phthalate aud starch acetate phthalate in buffers containing pancreatin (buffers A and C) do not differ significantly from the pooled disintegration times of the same tablets in buffers which no not contain pancreatin (buffers B and D). These data indicate that the enzymes contained in pancreatin U. S. P. do not aid in the dissolution of these enteric coatings at pH 6.9. (*b*) The pooled disintegration times of the tablets in buffers containing ox bile extract (buffers A and B) are significantly lower than the pooled disintegration times of the tablets in buffers which do not contain ox bile (buffers C and D). (*c*) There is no significant difference in buffers A and B with respect to the total of

TABLE III —ARTIFICIAL INTESTINAL FLUIDS, pH 6 9

Ingredient	Buffer A	Buffer B	Buffer C	Buffer D
Pancreatin U S P	10 0 Gm		10 0 Gm	.
Ox bile extract U. S P	4 0 Gm	4 0 Gm	.	
M/5 Potassium biphosphate	250 0 ml	250 0 ml.	250 0 ml.	250 0 ml.
M/5 Sodium hydroxide	140 0 ml (approx)	140 0 ml (approx)	140 0 ml (approx)	140 0 ml. (approx)
Deionized water *q s* to	1,000 0 ml	1,000 0 ml	1,000 0 ml.	1,000 0 ml
pH	6 9	6 9	6.9	6 9

TABLE IV.—DISINTEGRATION TIME OF TABLETS IN ARTIFICIAL INTESTINAL FLUIDS, pH 6 9

		Buffer			
		A	B	C	D
Lot		Average Disintegration Time ± Standard Deviation, (min)			
III (enteric coated)	Run 1	42 7 ± 2 1	43 8 ± 4 8	63 2 ± 4 0	59 0 ± 4 9
	Run 2	49 1 ± 2 0	42 3 ± 2 9	56 8 ± 2 3	55 1 ± 5 6
IV (enteric coated)	Run 1	42 8 ± 7 9	45 8 ± 2 1	48 7 ± 4 2	49 8 ± 6 9
	Run 2	45 4 ± 1 9	46 3 ± 3 8	51 3 ± 3 9	56 1 ± 2 9
III (subcoated)	Run 1	8 2 ± 0 8	8 3 ± 0 7	9 7 ± 0 8	13 8 ± 0 4
	Run 2	8 6 ± 0 8	8 3 ± 1 2	9 9 ± 1 4	10 7 ± 1 8
IV (subcoated)	Run 1	4 2 ± 0 2	4 4 ± 0 2	4 5 ± 0 1	4 7 ± 0 5
	Run 2	4 0 ± 0 3	4 0 ± 0 3	4 8 ± 0 9	4 0 ± 0 9

TABLE V —ANALYSIS OF VARIANCE OF T VALUES OF ENTERIC COATED TABLETS III AND IV

Source of Variation	d f.	M s	F Test
Total	95		...
Among lots	1	248 7	$F = \frac{248\ 7}{174\ 1} = 1\ 43$
Among buffers			
(A + C) − (B + D)	1	1 4	$F = <1$
(C + D) − (A + B)	1	2,516 4	$F = \frac{2516\ 4}{174\ 1} = 14\ 46^{a}$
A − B	1	2 8	$F = <1$
Interaction (buffers × lots)	3	174 1	$F = \frac{174\ 1}{57\ 1} = 3\ 05$
Between duplicate runs	8	57 1	$F = \frac{57\ 1}{17\ 4} = 3\ 28^{b}$
Within runs	80	17 4	

[a] Significant at 0 05 level [b] Significant at 0 01 level

the disintegration times of the enteric coated tablets in the buffers. (*d*) The variation in disintegration times between runs of six tablets is significantly greater than the variation within runs This has been observed with larger groups of enteric coated tablets (*e*) There is no significant difference between lots with respect to disintegration times in the four buffers

DISCUSSION

It is interesting to compare the standard deviations for runs of six tablets in the various buffers used in this study with those reported in the literature or calculated from data in the literature. Crisafio, Taylor, and Chatten (19) reported a very wide range of standard deviations calculated from the disintegration times of commercial enteric coated tablets in artificial intestinal fluids, pH 7 5 and 8 0, containing pancreatin but not containing ox bile. Brindamour and DeKay (20) reported disintegration data for a cellulose acetate phthalate-coated tablet and a commercial enteric coated tablet in various artificial gastric and intestinal fluids From their data standard deviations of six and twenty-three minutes were calculated for the cellulose acetate phthalate-coated and the commercial enteric-coated tablets, respectively, in their number 10 fluid[3] and standard deviations of seven and ten minutes were calculated for the cellulose acetate phthalate-coated and the commercial enteric-coated tablets, respectively, in their number 12 fluid.[4] The average of the standard deviations (based on 6 tablets and itemized in Table IV) for the enteric coated tablets in buffers A, B, C, and D are 3 5, 3 4, 3 6, and 5 1 minutes, respectively.

Both of these literature sources indicate that lowering the pH of the artificial intestinal fluids increases the range and hence the standard deviations of the disintegration times of enteric coated tablets Similar to the results reported in this paper Brindamour and DeKay (20) reported that the presence of

[3] Similar to buffer A in this report but their number 10 fluid contained calcium chloride and had a pH of 6 7

[4] The same as buffer A in this report except that their number 12 fluid contained calcium chloride

ox bile in the intestinal solutions caused the tablets to disintegrate more quickly; their data also indicate that pancreatin had no similar effect. In reviewing the literature on bile salts Haslewood (21) points out that recent work indicates bile salts form micelles in a way not fundamentally different from that well known in the case of detergents. Solubilization of fats, etc., in bile salt solutions may be explained on the theory of "solubilization" which has been applied to explain similar properties of detergents generally. The lowering of surface tension and the detergent action exerted by bile salts probably explains the action of ox bile extract in increasing the disintegration rate of enteric coated tablets.

SUMMARY AND CONCLUSIONS

1. Six new whole starch acetate phthalates and two new amylose acetate phthalates were synthesized.

2. Solubilities of these two compounds indicate that all but one of them would be useful substances for enteric coating. It was shown that the minimum pH at which the compounds dissolve is dependent upon the per cent free carboxyl content of the polymers.

3. Subcoated tablets were satisfactorily enteric coated with a starch acetate phthalate and with a commercial cellulose acetate phthalate.

4. Initial disintegration tests indicated that the tablets enteric coated with starch acetate phthalate did not have as high a resistance to artificial gastric juice, pH 1.2, as tablets enteric coated with cellulose acetate phthalate. There was no significant difference between the two lots of enteric coated tablets with respect to initial disintegration times in four artificial pancreatic fluids, pH 6.9.

5. It was shown that at pH 6.9 pancreatin U. S. P. had no influence on the disintegration rate of tablets enteric coated with starch acetate phthalate or cellulose acetate phthalate. It was shown that at pH 6.9 ox bile extract significantly increased the rate of disintegration of these enteric coated tablets. These data indicate that *in vitro* at pH 6.9 ionization of free carboxyl groups in these polymers is mainly responsible for the solution of the polymers and the disintegration of the enteric coatings.

6. The hypothesis is presented that the detergent action of bile salts in ox bile extract is responsible for the increased rate of disintegration of these coatings in the presence of ox bile extract.

7. Both lots of enteric coated tablets disintegrated completely in a phosphate buffer, pH 6.9, containing neither pancreatin nor ox bile extract.

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A Chromatographic Technique for the Identification of Fluorescein and Phenolphthalein Derivatives*

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New and simplified procedures are described for the chromatographic identification and separation of a number of fluorescein and phenolphthalein derivatives. The procedures employ phosphate buffers as a mobile phase. Methods are described for paper strip and cellulose column chromatography.

IN THE SYNTHESIS of fluorescein and phenolphthalein derivatives a number of by-products are formed. These products are difficult to separate by conventional means. As a result they are usually present to some extent in the final product. These compounds are weak acids and, as such, they are usually soluble in organic solvents and only slightly soluble in water. The salts of the same compounds have the opposite solubilities. This characteristic makes them unsuitable for general chromatographic

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techniques based on two phase systems. Partition methods can be used, however, as Lederer (1) and Graichen (2) have described identification techniques for phenolphthaleins and fluoresceins, respectively.

Experience in this laboratory has shown that partition methods tend to form long overlapping zones. In addition, a considerable amount of work and time is required for preparing the mobile phase. Some systems are not stable over a long period of time due to evaporation or esterification and are very sensitive to temperature changes.

The procedure described here is efficient and very simple. The solvents used are phosphate buffers. The advantages of this procedure are: (*a*) A variety of phosphate buffers may be kept for long periods of time if a few crystals of thymol are added to prevent the growth of microorganisms; (*b*) it doesn't require changing the atmosphere in the chromatography jar when a change in buffer system is desired; (*c*) the materials used are all inexpensive; (*d*) well defined zones are usually formed; (*e*) the identification can be completed in two hours; (*f*) variation between runs is very slight if equilibrating time and conditions are kept constant; (*g*) the same solvents can be used with cellulose columns to separate larger quantities; and (*h*) small temperature changes have a negligible effect on the results.

EXPERIMENTAL

Materials

Whatman No. 1 paper ($1^1/_2$ inches wide). Reagent grade Na_3PO_4. Reagent grade NaH_2PO_4. Chromatography jar 12 × 24 (descending type) with a stoppered hole in plate glass top. Micro-pipets (10 λ size). Whatman cellulose powder (ashless, standard grade). 14 × 800 mm. glass column. Automatic fraction collector (drop counting type).

Paper Chromatography

Preparation of Mobile Phase.—Two parent phosphate solutions were prepared—0.5 *M*[1] Na_3PO_4, and 0.5 *M* NaH_2PO_4. A portion of the Na_3PO_4 solution was placed in a beaker equipped with pH electrodes and a mechanical stirrer. The NaH_2PO_4 solution was added until the desired pH was reached. Several of the fluoresceins were separated using a 0.5 *M* solution of NaH_2PO_4 (reagent grade) as a mobile phase.

Preparation of Sample.—The samples were prepared as 1% solutions in 0.5 *M* Na_3PO_4.

Preparation of Chromatography Jar.—The type of jar used was of the descending type 12 × 24 inches. The plate-glass top was equipped with a stoppered hole such that the buffer could be added with a minimum of exposure. Two inches of water was kept in the bottom of the jar. Absorbent cotton or other wicking material was not used because such conditions would be difficult to reproduce.

Procedure.—Two λ (0.002 ml.) of the 1% sample solution was added to the paper strips. An attempt was made to keep the spots uniformly small. After the spots had dried the strips were placed in the jar. The top was replaced rapidly and they were allowed to equilibrate for one-half hour before the buffer was added. The strips were removed two hours later and allowed to air dry. They were then sprayed with a dilute solution of NaOH in 50% alcohol. The spots were then viewed in visible and ultraviolet light. The R_f values were determined for the point of greatest intensity.

Cellulose Column

Preparation of Column.—A chromatography column 14 × 800 mm. was used. It was equipped with a stopcock and a fritted glass disk as a cellulose support. A slurry of cellulose powder and water was poured into the column with 5 pounds of air pressure being applied after each of the small additions. A water level was maintained such that the slurry being added did not disturb the top of the column. After packing to a height of 700 mm. it was washed carefully with 0.5 *M* phosphate buffer. A buffer of pH 11.0 was used for phthaleins and pH 8.0 buffer was used for fluoresceins.

Operation of Column.—The sample to be chromatographed was prepared as a 0.5% solution in 0.5 *M* Na_3PO_4. On the top of the cellulose 0.02 cc. of the sample was placed. The chosen buffer was then added carefully such that the sample was washed into the column. Hydrostatic pressure was used

TABLE I.—R_f VALUES FOUND USING 0.5 *M* PHOSPHATE BUFFERS

Compound	pH 8.5	pH 11.0
Tetraiodofluorescein	0.01	..
Tetrabromofluorescein	0.05	..
Diiodofluorescein	0.11	..
Dibromofluorescein	0.12	..
Unsym. Dibromofluorescein	0.15	..
Diiodotetrachlorofluorescein	0.16	..
Nitrofluorescein[a]	0.25, 0.35	..
Fluorescein	0.41	..
Tetrabromophenoltetrabromophthalein	..	0.18
o-Phenylphenolphthalein	..	0.22
Dibromophenoltetrabromophthalein	..	0.34
Tetraiodophenoltetrachlorophthalein	..	0.35
Phenoltetrabromophthalein	..	0.43
Tetrabromophenoltetrachlorophthalein	..	0.45
Diiodophenoltetrachlorophthalein	..	0.48
Dibromo-*o*-phenylphenolphthalein	..	0.50
Dibromophenoltetrachlorophthalein	..	0.56
Phenoldibromophthalein	..	0.63
o-Cresoltetrachlorophthalein	..	0.65
Phenolphthalein	..	0.65

[a] Phenol condensed with 4-nitro phthalic acid yields two isomers.

[1] All molar solutions are in terms of sodium ion concentration.

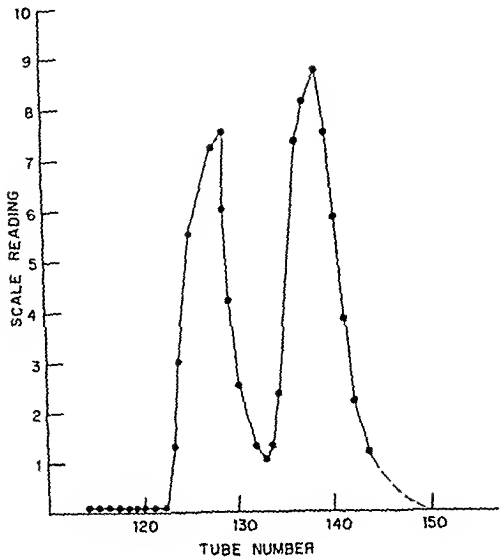

Figure 1.

to maintain a 40-cc. per hour rate of flow. An automatic fraction collector was used to collect 10 drops per tube.

Evaluation of Effluent.—Five cubic centimeters of distilled water was added to each tube and the optical density at 525 mμ determined on a Fisher Electrophotometer.

DISCUSSION

The R_f values given in Table I are approximate since no attempt was made to attain complete equilibration or to maintain a constant temperature. Results are easily reproduced, however, if the paper strips are equilibrated uniformly each time prior to adding buffer to the troughs. Several experiments were run on the fluoresceins using a wide range of buffers. This had little or no effect since the same R_f values were obtained at pH's of 8.0, 8 5, and 9.0. The effect of using more dilute buffers (0 05 → 0.005 *M*) was to cause tailing of the spots. The results obtained using 1.0, 0.5, and 0 1 *M* buffers were practically identical.

A cellulose column was used to separate the two isomeric nitrofluoresceins obtained by condensing 4-nitrophthalic acid and resorcinol. The buffer used was 0.5 *M* phosphate at pH 8.0. A procedure has been described for the separation of the aminofluorescein isomers, using *n*-butanol and cyclohexone with a column of buffered Hyflo-Supercel (3).

Figure 1 shows the results of applying a known solution of phenoltetrabromophthalein and phenoldibromophthalein to a cellulose column, using the procedure outlined in the experimental section.

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Some *in Vitro* Effects of Chlorpromazine, Lysergic Acid Diethylamide, and 5-Hydroxytryptamine on the Respiration of Rat Brain*

By WESLEY C. STARBUCK and HAROLD C. HEIM

Chlorpromazine, *in vitro*, produced a marked inhibition of oxygen uptake of brain homogenates while serotonin elicited only a slight inhibition. Lysergic acid diethylamide did not alter respiration nor did this compound evoke any effect on the inhibition caused by chlorpromazine or serotonin. It was found that the intraperitoneal administration of chlorpromazine did not alter brain serotonin levels and that brain from the treated animals respired at the same rate as that from untreated animals.

MANY years ago it was shown that certain drugs produce effects on oxidative processes of the brain, *in vitro* (1). Extension of this work by numerous investigators has led to the hypothesis that the mechanism of action of drugs which exert effects on the brain can, at least in part, be explained in terms of the effects produced by the drugs on enzymatic processes *in vivo*. In 1943 (2) it was observed that lysergic acid diethylamide (LSD) produced hallucinogenic effects. This compound represents only one of a series of drugs which have been known, for centuries, to produce mental aberrations following administration. The isolation of 5-hydroxytryptamine (5-HT) from brain (3) led to the suggestion that this compound might be, in some way, involved in brain function (4).

It has been postulated that mental aberrations induced by LSD might be due to a metabolic antagonism between LSD and 5-HT (5). I has been shown that the administration of r

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serpine is followed by a release of 5-HT in brain (6) and that the effects elicited on the central nervous system by either 5-HT or reserpine are blocked by LSD (7). Several investigators have reported that the administration of chlorpromazine does not cause the release of 5-HT (8). It has been reported that chlorpromazine has a potentiating effect upon the narcosis produced by barbiturates and by ethanol, and this same potentiating effect has been observed with 5-HT (9, 10). The same authors report that LSD antagonizes the potentiating effects of 5-HT on narcosis induced by barbiturates. It also has been found that LSD antagonizes the potentiating effect produced by chlorpromazine upon barbiturate-induced narcosis (10). Some studies have revealed that chlorpromazine inhibits oxygen uptake by brain, *in vitro* (11, 12), and the suggestion has been made that the barbiturates may elicit their effects on the brain by interfering with cellular metabolic processes (13).

Because the mechanisms by which 5-HT, LSD, and chlorpromazine produce their effects are not well understood, it was decided to study the effects of these drugs, singly and in combination, upon the respiration of brain homogenates.

EXPERIMENTAL

Adult Sprague-Dawley rats of either sex and weighing approximately 250 Gm. were used as the experimental animals in this study. Each animal was killed by decapitation after which the brain was immediately removed, washed, blotted dry, and weighed. The brain was then transferred to a glass homogenizer immersed in an ice bath and containing sufficient 0.05 M phosphate buffer, pH 7.4, so that 0.3 ml. of homogenate contained 50 mg. fresh tissue.

The substrate was a solution containing a mixture of pyruvate and fumarate prepared by dissolving the required amount of fumaric acid in sufficient 10 N NaOH to effect solution after which 50 ml. of distilled water was added. Sufficient sodium pyruvate was then added and the pH of the solution was adjusted to 7.4 with dilute HCl. The volume of the solution was then made to 100 ml. with distilled water. The substrate and reagents used in this study were identical to those used by Bernsohn (12).

The flasks contained 0.1 ml. 0.02 M ATP; 0.1 ml. 0.27 M $MgCl_2$; 0.3 ml. 0.23 M glucose; 0.3 ml. 10^{-5} M cytochrome C; 0.3 ml. 0.23 M KF; 0.3 ml. 0.03 M substrate; and 0.3 ml. homogenate. In those experiments where drugs were added to the flasks, 0.15 ml. 0.01 M chlorpromazine; 0.3 ml. 0.01 M 5-HT; or 0.3 ml. 10^{-3} M to 10^{-5} M LSD was added. The volume of fluid in all flasks was then adjusted to 3.0 ml. with 0.05 M phosphate buffer. In the experiments where endogenous respiration was determined, the fumarate-pyruvate mixture was replaced by buffer.

Oxygen consumption was measured by the direct method of Warburg. The temperature of the bath was 37°, the gas phase was air, and the equilibration period was ten minutes. At least eight animals were used for each experiment and all measurements were performed in triplicate.

To some of the animals chlorpromazine, 25 mg./Kg., was administered intraperitoneally. An hour after the administration of the drug these animals were sacrificed and the brains homogenized as outlined above.

In the experiments where the brains were used for determination of 5-HT as well as for measurement of oxygen uptake, two animals were sacrificed. Both brains were homogenized together in two volumes of cold distilled water. Equal volumes of this homogenate and buffer were mixed and used as before for the measurement of oxygen uptake. Enough homogenate was mixed with buffer so that at least six different flasks could be run. The rats used in alternate experiments were injected with chlorpromazine, 25 mg./Kg., intraperitoneally. The other runs were considered to be controls.

For the determination of 5-HT the method of Udenfriend, *et al.* (14), was used, samples being analyzed with the Aminco-Bowman Spectrofluorophotometer. Four and one-half milliliters of homogenate were used for each determination.

RESULTS AND DISCUSSION

All experimental results were subjected to statistical analysis and the standard error, E, determined from the equation $E = \sqrt{\Sigma d^2/[n(n-1)]}$. Results were considered to be significant if $m_1 - m_2 > 2\sqrt{E_1^2 + E_2^2}$. The results are reported in Table I.

TABLE I

Drug	Mm.³ O_2 Uptake/50 mg. Tissue/60 min.	Inhibition, %
None	171 ± 6	.
Chlorpromazine (5×10^{-4} M)	15 ± 1	91
Chlorpromazine (5×10^{-4} M) plus 5-HT (10^{-3} M)	33 ± 2	81
5-HT (10^{-3} M)	144 ± 3	16
LSD (10^{-4} M)	154 ± 8	10
Chlorpromazine (5×10^{-4} M) plus LSD (10^{-4} M)	13 ± 1	92
5-HT (10^{-3} M) plus LSD (10^{-4} M)	147 ± 4	14
Chlorpromazine (25 mg./Kg. injected intraperitoneally)	166 ± 7	3

It will be seen from Table I that chlorpromazine, at the stated concentration, produced approximately 90% inhibition of respiration, *in vitro.* This result confirms the finding of Bernsohn (12), who suggested that the inhibition of oxygen uptake is produced through interference, by chlorpromazine, with the hydrogen transport system. When chlorpromazine, 25 mg./Kg., was administered intraperitoneally to rats the oxygen uptake of homogenates prepared from the brains of these animals was not significantly different from that

obtained with homogenates prepared from brains of animals to which the drug had not been administered. The dose of chlorpromazine used was one-third of the reported 14-day LD_{50} (15) and was chosen because it was felt that such a high dose would be sufficient to demonstrate any effects on respiration due to chlorpromazine. The failure of the drug, at this dosage level, to exhibit inhibition of respiration would tend to cast doubt on the concept that the *in vivo* effects of chlorpromazine are due to an interference with the utilization of oxygen. If the assumption is made that the distribution of chlorpromazine in rat brain is similar to the distribution in dogs, as reported by Salzman and Brodie (16), the concentration in rat brain, an hour after the intraperitoneal administration of 25 mg./Kg. would be of the order of 3×10^{-8} M/100 mg. of brain. Bernsohn (12) found that concentrations greater than 10^{-4} M were necessary in order to demonstrate inhibition of oxygen uptake of brain, *in vitro*. The concentration of chlorpromazine necessary to produce inhibition of respiration, *in vitro* is, therefore, in excess of the usual therapeutic dose if a uniform distribution of the drug in the brain is assumed. It has been reported, however, that all of the areas of the brain are not equally susceptible to chlorpromazine (17), and that some areas may be more sensitive to this drug than are other areas.

The addition of 5-HT to the Warburg vessels produced a slight inhibition of oxygen uptake. Although the inhibition produced was found to be significant, it would seem improbable that so slight an inhibition could be considered the primary mechanism whereby 5-HT produces *in vivo* effects. When 5-HT was added to those flasks containing chlorpromazine, a slight reversal of the inhibitory effect of chlorpromazine was observed. It seems of interest to note that the contractile effect elicited by 5-HT on smooth muscle is antagonized by chlorpromazine (18). It has been shown, however, that reserpine appears to cause a release of 5-HT in brain (6) and that reserpine and chloropromazine produce similar, rather than antagonistic, effects on the central nervous system (9).

When LSD was added to the flasks no significant alteration in respiration was observed. This finding would be in agreement with the belief of Bain (19) but in opposition to the results obtained by Mayer-Gross, *et al.* (20), who reported that 10^{-9} M LSD produced a 30% stimulation of respiration, *in vitro*. These results would also tend to be in keeping with the evidence that LSD has no effect upon the duration of narcosis produced by barbiturates (7), especially since the interference with respiratory processes has been suggested as a mode of action of the barbiturates, *in vivo* (13). It was also noted during this study that the addition of LSD to flasks containing either 5-HT or chlorpromazine had no significant effect upon the inhibition of respiration produced by these two drugs. LSD is, however, able to reverse the potentiating action of 5-HT and of chlorpromazine upon the narcosis produced by barbiturates (10).

It was determined that the level of 5-HT in rat brain was approximately 4×10^{-3} μM per Gm. of brain. The previous intraperitoneal injection of chlorpromazine, 25 mg./Kg., produced no appreciable effect on the 5-HT levels. This finding corroborates that of Brodie (8), who found that chlorpromazine does not cause the release of 5-HT in the brain.

SUMMARY

1. The effects of chlorpromazine, 5-HT, and LSD on the oxygen uptake of rat brain homogenate have been studied.

2. Chlorpromazine was found to produce a marked inhibition of oxygen uptake. 5-HT produced a slight inhibitory effect.

3. LSD produced no significant effect upon the respiration of the homogenates.

4. The inhibition elicited by chlorpromazine or by 5-HT was not altered by LSD.

5. Chlorpromazine, when administered intraperitoneally, elicited no significant effect on respiration of the homogenates.

6. Chlorpromazine, when administered intraperitoneally, did not alter the concentration of 5-HT in the brain.

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Convulsant Properties of Oral Pentylenetetrazol Mixtures*

By EUGENE A. CONRAD†, J. MAXWELL LITTLE, and GEORGE H. ARMSTRONG

Mice received during a three-day period oral pentylenetetrazol, reserpine, and niacin singly or in combination prior to challenge with oral pentylenetetrazol. Niacin (20 mg./Kg./day) and pentylenetetrazol (50 mg./Kg./day) did not significantly influence the challenge CD_{50} and TD_{50}. Reserpine (8 mg./Kg./day) induced an apparent lowering of CD_{50} and a significant decrease in TD_{50}; dietary limitation led to similar results. The administration of a mixture containing the above concentrations of pentylenetetrazol and niacin, and reserpine 0.5 mg./Kg./day did not significantly affect the subsequent challenge CD_{50} and TD_{50}.

Thompson and Proctor (1) observed a clinical improvement in 46 of 60 elderly psychiatric patients treated with subconvulsive dosages of pentylenetetrazol-niacin mixtures. The potent tranquillizing action of reserpine suggested the addition of this drug might improve the therapeutic effectiveness of the above mixture. Although reserpine possesses ataractic properties, Chen and Bohner (2), and others, reported a lowered threshold for pentylenetetrazol convulsions following premedication with toxic doses of reserpine. For this reason, the present experiments were designed to study the influence of oral reserpine alone, or in combination with pentylenetetrazol and niacin, on the convulsant seizures induced subsequently by challenge with oral pentylenetetrazol.

MATERIALS AND METHODS

Healthy female albino mice weighing 18–25 Gm. were used throughout this study. The animals were fed *ad libitum* except during the times noted in the dosage schedule.

Pentylenetetrazol and niacin were dissolved in physiological saline solution in such concentrations that 1 cc. would be administered by stomach tube per 25 Gm. of animal weight. Reserpine or it's combinations were prepared similarly by suspension in 0.5% aqueous methylcellulose solution using a glass Potter-Elvehjem homogenizer.

During the three-day premedication period the mice were fasted each day for three hours (9 a. m.–noon) and then given, orally, one-half the daily dose of reserpine, or pentylenetetrazol, or a combination of both with niacin; the fast was continued until 4 p. m. when the oral administration was repeated and the animals allowed to eat *ad libitum*. Such regimens led to a significant change in final body weight; therefore, the influence of dietary restriction alone was also investigated. One group of mice was permitted access to a limited amount of food in order to lead to a weight loss of 15–20% during a three-day period.

On the fourth day of the experimental period, the mice were fasted for three hours and then challenged with varying doses of oral pentylenetetrazol; a minimum of 10 mice were employed for each dosage. All mice were observed for thirty minutes following challenge. The numbers of animals showing clonic and tonic convulsions were noted and the results analyzed by the method of Litchfield and Wilcoxon (3).

RESULTS

Premedication with oral pentylenetetrazol, niacin, or reserpine led to significant changes in final body weight as noted in Table I. The subacute administration of a subthreshold dose of pentylenetetrazol induced a gain while the other regimens resulted in a loss in body weight.

Previous treatment with subconvulsive amounts of pentylenetetrazol or niacin did not influence significantly the CD_{50} or TD_{50} of pentylenetetrazol challenge. Reserpine (0.5 mg./Kg./day) administered alone induced a lowering of TD_{50} (borderline significance); however, the mixture containing this amount of reserpine did not significantly influence the TD_{50}. The higher dose of reserpine (8 mg./Kg./day) led to an apparent lowering of CD_{50} and significant decrease in TD_{50}.

The limitation of daily dietary intake resulted in a significantly lowered threshold for tonic convulsions and an apparent decrease in CD_{50}.

DISCUSSION

The data presented confirm the previous observation of a lowered threshold for pentylenetetrazol tonic seizures by subacute premedication with toxic dosages of reserpine. Jenney and Pfeiffer (4) reported a 46% decrease in pentylenetetrazol threshold following dosing with reserpine (50 mg./Kg./day) for nine days; in the present study, premedication with 8 mg./Kg./day for three days led to a 34% diminution in TD_{50}. This alteration in threshold may be due, in part, to the accompanying metabolic changes (as reflected in body weight loss) resulting from reserpine toxicity; the percentage lowering of TD_{50} noted following dietary limitation closely resembles that noted after premedication with reserpine 8 mg./Kg./day.

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TABLE I.—THE INFLUENCE OF SUBACUTE PREMEDICATION ON CONVULSIONS INDUCED BY ORAL PENTYLENETETRAZOL

Daily Regimen	Change in Body Weight, %	Clonic $Dose_{50}$	Tonic $Dose_{50}$
Control (131)[a]	+0 7(±0 8)[b]	89(78–101)[c]	134(120–149)
P-50[d] (40)	+5 4(±1 1) P<0 01	108(92–127) P>0 05	142(123–163) P>0 05
N-20[e] (47)	−4 2(±1 0) P<0 01	85(70–104) P>0 05	134(112–160) P>0 05
R-0 5[f] (47)	−6 1(±0 9) P<0 01	85(75–96) P>0 05	113(101–126) P = 0.05
R-8 (40)	−10 6(±1 3) P<0 01	66(51–86) P = 0 05	88(76–103) P<0.05
P, N, R-0 5 (75)	−4 3(±0 7) P<0 01	78(64–95) P>0.05	125(111–141) P>0.05
Diet limitation (49)	−19 4(±0 8) P<0 01	69(60–80) P<0 05	83(70–99) P<0.05

[a] Number of animals tested

[b] Mean values ± S E as computed by $S\ D = \sqrt{\frac{\Sigma(x - m)^2}{N}}$ and $S\ E. = \frac{S\ D.}{\sqrt{N}}$ probability values determined from Fisher's table of t

[c] mg /Kg body weight, confidence limits for 19/20 cases in parentheses

[d] Pe[illegible] /day)

[e] N[illegible]

[f] R[illegible]

Premedication with reserpine (0 5 mg /Kg /day) alone or admixed with niacin and pentylenetetrazol failed to induce a highly significant lowering of CD_{50} or TD_{50} This concentration of reserpine represents an amount greater than the maximal chronic oral tolerated doses noted in mice by Schneider (5); the latter was based on changes in growth rate and survival time (6) Also, it has been observed in clinical trials that chronic administration of an oral mixture of pentylenetetrazol, niacin, and reserpine to senile psychotics did not precipitate convulsive seizures (7).

An apparent lowering of CD_{50} was noted in mice receiving a limited diet or reserpine 8 mg /Kg./day. Many of these animals showed, initially, several clonic jerks followed almost immediately by a full tonic extension of the hind limbs However, Chen and Bohner (2) did not observe a potentiation of clonic seizures in mice premedicated with reserpine (8 mg /Kg intraperitoneally) and challenged with intravenous pentylenetetrazol

SUMMARY

Subacute premedication with an oral mixture containing pentylenetetrazol, niacin, and a therapeutic dose of reserpine did not significantly influence the CD_{50} or TD_{50} of subsequent challenge with oral pentylenetetrazol. A limitation of dietary intake sufficient to induce a loss of 15–20 per cent in final body weight induced a significant lowering of pentylenetetrazol TD_{50}

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By EUGENE A. CONRAD†, J. MAXWELL LITTLE, and GEORGE H. ARMSTRONG

Mice received during a three-day period oral pentylenetetrazol, reserpine, and niacin singly or in combination prior to challenge with oral pentylenetetrazol. Niacin (20 mg./Kg./day) and pentylenetetrazol (50 mg./Kg./day) did not significantly influence the challenge CD_{50} and TD_{50}. Reserpine (8 mg./Kg./day) induced an apparent lowering of CD_{50} and a significant decrease in TD_{50}; dietary limitation led to similar results. The administration of a mixture containing the above concentrations of pentylenetetrazol and niacin, and reserpine 0.5 mg./Kg./day did not significantly affect the subsequent challenge CD_{50} and TD_{50}.

THOMPSON AND PROCTOR (1) observed a clinical improvement in 46 of 60 elderly psychiatric patients treated with subconvulsive dosages of pentylenetetrazol-niacin mixtures. The potent tranquillizing action of reserpine suggested the addition of this drug might improve the therapeutic effectiveness of the above mixture. Although reserpine possesses ataractic properties, Chen and Bohner (2), and others, reported a lowered threshold for pentylenetetrazol convulsions following premedication with toxic doses of reserpine. For this reason, the present experiments were designed to study the influence of oral reserpine alone, or in combination with pentylenetetrazol and niacin, on the convulsant seizures induced subsequently by challenge with oral pentylenetetrazol.

MATERIALS AND METHODS

Healthy female albino mice weighing 18–25 Gm. were used throughout this study. The animals were fed *ad libitum* except during the times noted in the dosage schedule.

Pentylenetetrazol and niacin were dissolved in physiological saline solution in such concentrations that 1 cc. would be administered by stomach tube per 25 Gm. of animal weight. Reserpine or it's combinations were prepared similarly by suspension in 0.5% aqueous methylcellulose solution using a glass Potter-Elvehjem homogenizer.

During the three-day premedication period the mice were fasted each day for three hours (9 a. m.–noon) and then given, orally, one-half the daily dose of reserpine, or pentylenetetrazol, or a combination of both with niacin; the fast was continued until 4 p. m. when the oral administration was repeated and the animals allowed to eat *ad libitum*. Such regimens led to a significant change in final body weight; therefore, the influence of dietary restriction alone was also investigated. One group of mice was permitted access to a limited amount of food in order to lead to a weight loss of 15–20% during a three-day period.

On the fourth day of the experimental period, the mice were fasted for three hours and then challenged with varying doses of oral pentylenetetrazol; a minimum of 10 mice were employed for each dosage. All mice were observed for thirty minutes following challenge. The numbers of animals showing clonic and tonic convulsions were noted and the results analyzed by the method of Litchfield and Wilcoxon (3).

RESULTS

Premedication with oral pentylenetetrazol, niacin, or reserpine led to significant changes in final body weight as noted in Table I. The subacute administration of a subthreshold dose of pentylenetetrazol induced a gain while the other regimens resulted in a loss in body weight.

Previous treatment with subconvulsive amounts of pentylenetetrazol or niacin did not influence significantly the CD_{50} or TD_{50} of pentylenetetrazol challenge. Reserpine (0.5 mg./Kg./day) administered alone induced a lowering of TD_{50} (borderline significance); however, the mixture containing this amount of reserpine did not significantly influence the TD_{50}. The higher dose of reserpine (8 mg./Kg./day) led to an apparent lowering of CD_{50} and significant decrease in TD_{50}.

The limitation of daily dietary intake resulted in a significantly lowered threshold for tonic convulsions and an apparent decrease in CD_{50}.

DISCUSSION

The data presented confirm the previous observation of a lowered threshold for pentylenetetrazol tonic seizures by subacute premedication with toxic dosages of reserpine. Jenney and Pfeiffer (4) reported a 46% decrease in pentylenetetrazol threshold following dosing with reserpine (50 mg./Kg./day) for nine days; in the present study, premedication with 8 mg./Kg./day for three days led to a 34% diminution in TD_{50}. This alteration in threshold may be due, in part, to the accompanying metabolic changes (as reflected in body weight loss) resulting from reserpine toxicity; the percentage lowering of TD_{50} noted following dietary limitation closely resembles that noted after premedication with reserpine 8 mg./Kg./day.

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TABLE I.—THE INFLUENCE OF SUBACUTE PREMEDICATION ON CONVULSIONS INDUCED BY ORAL PENTYLENETETRAZOL

Daily Regimen	Change in Body Weight, %	Clonic $Dose_{50}$	Tonic $Dose_{50}$
Control (131)[a]	+0.7(±0.8)[b]	89(78–101)[c]	134(120–149)
P-50[d] (40)	+5.4(±1.1) P<0.01	108(92–127) P>0.05	142(123–163) P>0.05
N-20[e] (47)	−4.2(±1.0) P<0.01	85(70–104) P>0.05	134(112–160) P>0.05
R-0.5[f] (47)	−6.1(±0.9) P<0.01	85(75–96) P>0.05	113(101–126) P = 0.05
R-8 (40)	−10.6(±1.3) P<0.01	66(51–86) P = 0.05	88(76–103) P<0.05
P, N, R-0.5 (75)	−4.3(±0.7) P<0.01	78(64–95) P>0.05	125(111–141) P>0.05
Diet limitation (49)	−19.4(±0.8) P<0.01	69(60–80) P<0.05	83(70–99) P<0.05

[a] Number of animals tested.

[b] Mean values ± S.E. as computed by $S.D. = \sqrt{\frac{\Sigma(x - m)^2}{N}}$ and $S.E. = \frac{S.D.}{\sqrt{N}}$ probability values determined from Fisher's table of t.

[c] mg./Kg. body weight, confidence limits for 19/20 cases in parentheses.

[d] Pentylenetetrazol (50 mg./Kg./day).

[e] Niacin (20 mg./Kg./day).

[f] Reserpine (0.5 mg./Kg./day).

Premedication with reserpine (0.5 mg./Kg./day) alone or admixed with niacin and pentylenetetrazol failed to induce a highly significant lowering of CD_{50} or TD_{50}. This concentration of reserpine represents an amount greater than the maximal chronic oral tolerated doses noted in mice by Schneider (5); the latter was based on changes in growth rate and survival time (6). Also, it has been observed in clinical trials that chronic administration of an oral mixture of pentylenetetrazol, niacin, and reserpine to senile psychotics did not precipitate convulsive seizures (7).

An apparent lowering of CD_{50} was noted in mice receiving a limited diet or reserpine 8 mg./Kg./day. Many of these animals showed, initially, several clonic jerks followed almost immediately by a full tonic extension of the hind limbs. However, Chen and Bohner (2) did not observe a potentiation of clonic seizures in mice premedicated with reserpine (8 mg./Kg. intraperitoneally) and challenged with intravenous pentylenetetrazol.

SUMMARY

Subacute premedication with an oral mixture containing pentylenetetrazol, niacin, and a therapeutic dose of reserpine did not significantly influence the CD_{50} or TD_{50} of subsequent challenge with oral pentylenetetrazol. A limitation of dietary intake sufficient to induce a loss of 15–20 per cent in final body weight induced a significant lowering of pentylenetetrazol TD_{50}.

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A Note on the Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) II*

Catharanthine, Lochnericine, Vindolinine, and Vindoline

By MARVIN GORMAN, NORBERT NEUSS, GORDON H SVOBODA, ALBERT J. BARNES, Jr, and NANCY J. CONE

VARIOUS INVESTIGATORS have isolated from *Vinca rosea* Linn the known alkaloids ajmalicine (1–4), akuammine (2), tetrahydroalstonine (1, 4), serpentine (3, 4), lochnerine (1–3), and reserpine (5) In addition to these the new alkaloids, vindoline (6) and vincaleucoblastine (7), have been reported In an earlier communication one of us has confirmed the presence of tetrahydroalstonine, ajmalicine, and lochnerine and reported the isolation of three new compounds leurosine, perivine, and virosine (1)

Since that time we have been able to isolate from this plant[1] three additional, apparently new alkaloids catharanthine, lochnericine, and vindolinine, as well as confirming the presence of vincaleucoblastine, serpentine, and vindoline

The present note describes the characterization of these three new alkaloids as well as the partial structure of vindoline

Chromatography of a larger batch (1) of the weakly basic fraction yielded the following alkaloids in the order of their elution catharanthine, vindolinine, tetrahydroalstonine, ajmalicine, vindoline, leurosine, vincaleucoblastine, and virosine[2] Lochnericine is obtained by the chromatography of an alkaloidal fraction prepared from a hexane extract of the plant Serpentine was isolated as its nitrate by chromatography of the strong bases obtained from an alkaline benzene extract (1)

Catharanthine crystallizes from methanol, m p 126–128°, $[\alpha]_D^{27} = +29\ 8°$ ($CHCl_3$), pK'a = 6 8 (66% DMF), and is a $C_{21}H_{24}O_2N_2$ compound It forms different solvates depending upon conditions accompanying crystallization and drying

Anal —Calcd for $C_{21}H_{24}O_2N_2\ H_2O$ C, 71 16, H, 7 39, N, 7 90 Found C, 71 02, H, 7 38, N, 7 71

Its sulfate recrystallized from alcohol, m p 164–167° (decompn)

Anal —Calcd for $C_{21}H_{24}O_2N_2\ {}^1/_2H_2SO_4\ {}^1/_2H_2O$ C, 63 94, H, 6 64, N, 7 10, S, 4 06 Found C, 64 03, H, 6 76, N, 6 91, S, 3 90

The two oxygen atoms of catharanthine are contained in a carbomethoxyl moiety as evidenced by the reduction with lithium aluminum hydride to give catharanthinol, m p 227–228° (decompn) from methanol

Anal —Calcd for $C_{20}H_{24}ON_2$ C, 77 88, H, 7 84, N, 9 08, mol wt, 308 4 Found C, 77 73, H, 7 85, N, 8 85, mol wt, 309 3 (X ray data)

The base readily forms a methiodide with excess methyl iodide in benzene Crystallization from methanol yields material m p 232–234° (decompn)

Anal —Calcd for $C_{21}H_{24}O_2N_2\ CH_3I$ C, 55 23, H, 5 69, N, 5 86, I, 26 53 Found C, 55 07, H, 5 63, N, 5 89, I, 26 71

The ultraviolet spectrum of catharanthine is typical of an unsubstituted (8) indole alkaloid λ_{max}^{EtOH} 226 mμ (log a_M 4 56), 284 mμ (log a_M 3 92), 292 mμ (log a_M 3 88), λ_{min}^{EtOH} 250 mμ (log a_M 3 39)

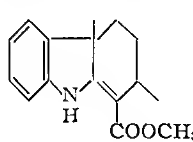
Its infrared spectrum has the following characteristic bands $\lambda_{max}^{CHCl_3}$ 2 92, 5 81, 7 92, 9 29 μ Catharanthine readily absorbs one mole of hydrogen with PtO_2 catalyst in alcohol, indicating an isolated double bond Thus the empirical formula of this alkaloid allows the assignment of its nucleus to a pentacyclic fused ring system with the partial formula I

$$C_{19}H_{21}N_2(COOCH_3)$$

I

Lochnericine crystallized from methanol, m p 190–193° (decompn), $[\alpha]_D^{27} = -432°$ ($CHCl_3$), pK'a = 4 2 (66% DMF)

Anal —Calcd for $C_{21}H_{24}O_3N_2$ C, 71 57 H, 6 86, N, 7 95, mol wt, 352 4, (1) OCH_3, 8 86 Found C, 71 49, 71 43 H, 6 97, 6 71, N, 8 10, mol wt, 352 (X ray data), (1) OCH_3, 9 10



II

The spectral properties of lochnericine, as well as the high negative value for its rotation, indicate that this alkaloid contains the same type of chromophore (II) as tabersonine (9), akuammicine (10), and echitamidine (9)

The ultraviolet spectrum is characterized by the following absorption λ_{max}^{EtOH} 226 mμ (log a_M 4 00), 297 mμ (log a_M 4 04), 327 mμ (log a_M 4 23), λ_{min}^{EtOH} 215 mμ (log a_M 3 90), 256 mμ (log a_M 2 86), 305 mμ (log a_M 4 03)

The infrared spectrum in $CHCl_3$ solution shows the following prominent bands $\lambda_{max}^{CHCl_3}$ 2 96, 3 40, 3 59, 5 96, 6 19, 7 97 μ

Vindolinine could not be induced to crystallize as a free base Its dihydrochloride crystallizes readily from methanol ether, m p 210–212 (decompn), $[\alpha]_D^{27} = -8°$ (H_2O), pK'a = 3 3, 7 1 (66% DMF)

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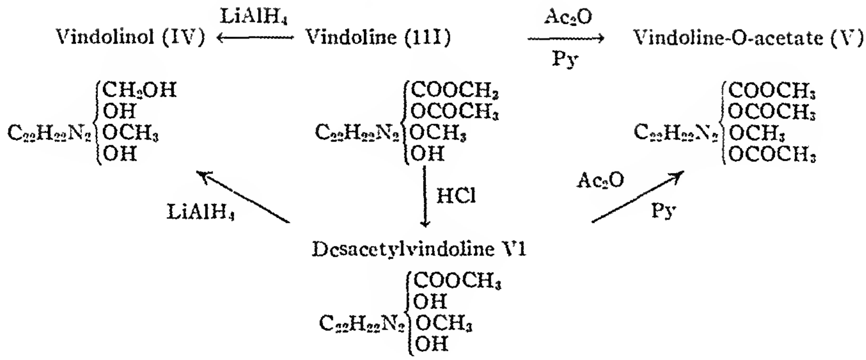
* Received March 17, 1959 from Lilly Research Laboratories and Organic Chemical Development Eli Lilly and Co Indianapolis Ind

The authors are grateful to Dr H E Boaz Mr Lee Howard and Miss Martha Hofmann for spectral data and electrometric titrations Miss Ann Van Camp for X ray data Miss Ruthanne Miller and Messrs R J Armstrong A T Oliver and George Johnson for laboratory assistance and Messrs W L Brown R Hughes H L Hunter and G M Maciak for microanalyses We should like to thank Dr Noble for an authentic sample of vincaleucoblastine sulfate and Drs Janot and Le Men for their sample of vindolinine They have isolated this compound independently from us and will publish their results elsewhere The name of the compound however has been chosen by mutual agreement

[1] The drug used in this study was obtained from Meer Corp and was botanically authenticated by Dr V H Madis

[2] All known alkaloids were identified by comparison of melting points X ray powder diffraction patterns infrared and ultraviolet spectra with those of the authentic specimen



The ultraviolet spectrum is typical of a dihydroindole alkaloid (11): $\lambda_{max.}^{EtOH}$ 245 mμ (log a_M 3.83), 300 mμ (log a_M 3.37); $\lambda_{min.}^{EtOH}$ 271 mμ (log a_M 2.98).

The infrared spectrum shows the following characteristic bands: $\lambda_{max.}^{Nujol}$ 5.77, 6.43, 8.21 μ.

Anal.—Calcd. for $C_{21}H_{24-26}O_2N_2 \cdot 2HCl$: C, 61.61; 61.31; H, 6.40; 6.86; N, 6.84; 6.81; Cl, 17.32; 17.24. Found: C, 61.54; 61.44; H, 6.56; 6.58; N, 6.58; 6.64; Cl, 17.03.

It forms a picrate which, when recrystallized from acetone-water, melts at 268–272° (decompn.).

Anal.—Calcd. for $C_{21}H_{24-26}O_2N_2 \cdot C_6H_3O_7N_3$: C, 57.34; 57.14; H, 4.81; 5.15; N, 12.38; 12.34. Found: C, 57.01; H, 4.77; N, 12.31.

Vindoline crystallizes from ether, m. p. 154–155°, $[\alpha]_D^{27}$ = +42° ($CHCl_3$), pK'a = 5.5 (66% DMF), and is a $C_{25}H_{32}O_6N_2$ compound.

Anal.—Calcd. for $C_{25}H_{32}O_6N_2$: C, 65.77; H, 7.07; N, 6.14. Found: C, 65.99; H, 7.02; N, 6.04.

Its hydrochloride, recrystallized from acetone, melts at 161–164°.

Anal.—Calcd. for $C_{25}H_{32}O_6N_2 \cdot HCl$: C, 60.90; H, 6.75; N, 5.68; Cl, 7.19. Found: C, 61.17; H, 6.83; N, 5.77; Cl, 6.99.

The ultraviolet absorption spectrum of the alkaloid suggests a dihydroindole moiety (11): $\lambda_{max.}^{EtOH}$ 212 mμ (log a_M 4.49), 250 mμ (log a_M 3.74), 304 mμ (log a_M 3.57); $\lambda_{min.}^{EtOH}$ 241 mμ (log a_M 3.71), 274 mμ (log a_M 3.02).

Its infrared spectrum contains the following characteristic bands: $\lambda_{max.}^{CHCl_3}$ 3.37, 3.42, 3.51, 3.56, 3.7–4.1 (shoulder), 5.74, 6.20, 8.1, 9.7, 12.0, 12.2 μ.

Vindoline readily forms an O-acetate with pyridine in Ac_2O, m. p. 127–128.5°[3]; disappearance of shoulder 3.7–4.1 in the infrared spectrum.

Anal.—Calcd. for $C_{27}H_{34}O_7N_2$: C, 65.04; H, 6.87; N, 5.62; CH_3CO (2), 17.27. Found: C, 65.03; H, 6.99; N, 5.43; CH_3CO (2), 17.45.

Lithium aluminum hydride reduction in a mixture of ether and tetrahydrofuran yields the corresponding alcohol, vindolinol.

The infrared spectrum showed no carbonyl absorption and two new hydroxyl bands were present: $\lambda_{max.}^{CHCl_3}$ 2.84, 2.95 μ, m. p. 172–176°.[3]

Anal.—Calcd. for $C_{22}H_{30}O_4N_2$: C, 68.34; H, 7.82; N, 7.82. Found: C, 68.46; H, 7.85; N, 7.40.

Treatment of vindoline with concentrated hydrochloric acid (eight minutes, reflux) removes the acetyl group with formation of desacetylvindoline, m. p. 156–157°[3], $\lambda_{max.}^{CHCl_3}$ 2.84 μ.

Anal.—Calcd. for $C_{23}H_{30}O_5N_2$: C, 66.64; H, 7.30; N, 6.76. Found: C, 66.61; H, 7.37; N, 6.83.

Reduction of desacetylvindoline with lithium aluminum hydride yields vindolinol, while acetylation with acetic anhydride in pyridine gives vindoline-O-acetate.

Vindoline readily absorbs one mole of hydrogen with PtO_2 in alcohol indicating an isolated double bond.

Simultaneous determination of O-CH_3 and (N)-CH_3 (Zeisel) on vindoline and vindolinol gave the following results:

Vindoline; *Anal.*—Calcd. for N-CH_3 (1), 3.29; O-CH_3 (2), 13.6. Found: N-CH_3, 1.29; O-CH_3, 18.29.

Vindolinol; *Anal.*—Calcd. for N-CH_3 (1), 3.89; O-CH_3 (1), 8.03. Found: N-CH_3, 3.53; O-CH_3, 11.29.

Since the conversion of N- to O-methyl is known (12), the results above indicate the presence of one N-CH_3 group and one O-CH_3 ether in vindoline and its derivatives.

Analyses of vindoline, its salts, and transformation products allow establishing of partial structures for these compounds (III–VI) and clearly indicate that vindoline is a $C_{25}H_{32}O_6N_2$ compound containing a fused five-ring system, rather than a $C_{27}H_{34}O_7N_2$ as postulated by Kamat, *et al.* (6).

The work toward the structure elucidation of these alkaloids, now in progress in these laboratories, will be published later.

[3] Ultraviolet spectrum like that of vindoline.

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Book Notices

Scale-Up in Practice. Edited by RICHARD FLEMING. Reinhold Publishing Corp., New York, 1958. iv + 134 pp. 12.5 x 18.5 cm. Price $4.50.

Pilot plant problems are discussed in this book. Chapters are included on the factors justifying the operation of pilot plants; a brief review of scale-up theory; simulating operation of processes and equipment with computers; the hazards involved in scale-up; the process and business economics of scale-up; and organizing for scale-up.

Psychopharmacology Frontiers. Edited by NATHAN S. KLINE. Little, Brown and Co., Boston, 1959. xxiii + 533. 15.5 x 23.5 cm. Price $10.

This book is composed of a series of papers by experts in their respective fields which were presented to a Psychopharmacology Symposium of the Second International Congress of Psychiatry, held in Zurich, Switzerland, on September 2–4, 1957. This volume should serve as a reference book and as a working guide to the compex field of psychopharmacology.

Psychotherapeutic Drugs. By ASHTON L. WELSH. Charles C Thomas, Springfield, 1958. xiii + 139 pp. 15 x 22.5 cm. Price $4.75.

This monograph is designed to describe the various psychotherapeutic agents, their indications for use, and their side effects. The book is divided into five parts, each part devoted to a discussion of one of the major groups of tranquilizers including phenothiazines, rauwolfia alkaloids, substituted propanediols, diphenylmethane derivatives, and ureides and amides.

The Evaluation of Drug Toxicity. A Symposium. Edited by A. L. WALPOLE and A. SPINKS. Little, Brown and Co., Boston, 1958. xi + 138 pp. 15.5 x 23.5 cm.

Included in this book is a group of papers presented to a symposium held in October 1957 in observance of the dedication of the new research laboratories of Imperial Chemical Industries (Pharmaceutical Division). Many phases of the evaluation of drug toxicity are discussed.

Acetophenetidin. By PAUL K. SMITH. Interscience Publishers, New York, 1958. x + 180 pp. 15.5 x 23.5 cm. Price 5.75.

This monograph is the fourth in a series of critical reviews of the literature on analgesic and sedative drugs. The first volume dealt with acetanilid, the second with salicylates, and the third with antipyrine. In the monograph on acetophenetidin, a critical bibliographic review is presented which makes available all of the pertinent literature in a convenient form. It should serve as an aid to future investigators by calling their attention to the inadequacies of past work so that these may be a guide to planning more definitive experiments. Acetaminophen (*N*-acetyl-*p*-aminophenol), the major metabolite of acetophenetidin is believed to be responsible for the therapeutic activity of acetophenetidin. Because of the close relationship existing between these two drugs, the author has included in this volume many references to the increasing number of studies dealing with *N*-acetyl-*p*-aminophenol.

Advances in Pest Control Research. Vol. II. Edited by R. L. METCALF. Interscience Publishers, New York, 1958. vii + 426 pp. 15 x 2 cm. Price $12.50.

This is the second in a series of selected contributions by specialists in fields related to pest control research. The first volume of the series was reviewed in THIS JOURNAL, 47, 230(1958). The scope of Volume II is indicated by the chapter titles which are: The fluid kinetics of application of pesticidal chemicals; Innate toxicity of fungicides; Isotope dilution techniques for the determination of pesticide residues; Wool digestion and mothproofing; The relation of chemical structure to activity for the 2,4-D-type herbicide and plant growth regulator; Chemical structure and activity of DDT analogues with special consideration of their spatial structures; and The spread of insecticide resistance in pest species.

Bacteriophages. By MARK H. ADAMS. Interscience Publishers, New York, 1959. xviii + 592 pp. 15 x 23 cm. Price $15.

Phage research has had an erratic history, and this is probably the first comprehensive book on bacteriophages. It should be useful to every student of modern biology.

Reversible Renal Insufficiency. Diagnosis and Treatment. By DONALD H. ATLAS and PETER GABERMAN. The William and Wilkins Co., Baltimore, 1958. ix + 233 pp. 14.5 x 21.5 cm. Price $7.

This monograph on reversible acute and chronic renal insufficiency is intended to be of particular value to nephrologists, physiologists, and internists, as well as to general practitioners and medical students. It is a comprehensive review of the specific field.

Year Book of Drug Therapy. 1958–59 Series. Edited by HARRY BECKMAN. The Year Book Publishers, Chicago, 1959. 569 pp. 13 x 19.5 cm. Price $7.50.

This volume represents Dr. Beckman's review of medical literature for the "series year" beginning September 1958. His concise, informative reporting is up to his established high standards. Occasional editorial comments, such as his criticism of poor trial design for clinical testing of new neuropsychiatric tranquilizers and stimulants, are interesting. The series is a useful reflection of therapeutic progress, and is a valuable reference collection. Good indexes are appended.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII | *MAY 1959* | NUMBER 5

Drug Addiction II*

Variation of Addiction

By *JOHN R. NICHOLS*† and *W. MARVIN DAVIS*

As in previous experiments with this procedure, relapse by animals was demonstrated. Different degrees of drug-directed behavior were produced in groups of rats which underwent escape training. Small injections given between training trials increased the drug-directed behavior, probably because they retarded, but did not prevent, some loss of tolerance and dependence during the procedure. It seems likely that the time interval between training trials is too long and should be shortened for maximal effect on drug-seeking behavior. More important, this experiment indicates that for rats, as for man, the conditions under which morphine is received is more highly related to the development of drug-seeking behavior than is any "inherent rewarding properties" of morphine.

THE DEVELOPMENT of compulsive drug-seeking behavior in man seems related to whether he takes an active role in using the drug. Illegal opiate-users initiate the drug-taking action and this behavior rapidly becomes a chronic obsessive-compulsion. This is not true for the passive recipient of opiates in a hospital setting.

Behavioral techniques which permit animals an active role in opiate use are among the newest methods for studying opiate addiction. Chimpanzees (1) and rats (2) have been shown to develop drug-seeking behavior when suffering withdrawal symptoms. Recently, Nichols, Headlee, and Coppock (3) have shown that escape-training can cause rats to increase their performance of an instrumental act of escape, i.e., drinking a previously rejected morphine solution. Beach (4) has shown that rats develop a preference for stimuli associated with the effects of morphine injection. In both of these experiments, the changes in behavior wrought by the experimental procedure survived complete abstinence from morphine for several weeks.

The explanation of these drug-directed behavioral patterns in the rat has been based upon learning-conditioning principles. Ample evidence, e. g., Skinner (5), Verplanck (6), Thorndike (7), Mowrer (8), and many other conditioning studies (9, 10), supports the following principles: any response which is reinforced tends to be learned and repeated; and the termination of a disagreeable or noxious stimulus (*e. g.*, electric shock, immersion in water, or excessive heat or cold) is a reinforcing circumstance.

If a response is reinforced by the termination of a noxious stimulus, the procedure is called "escape training" and the response is called the "instrumental act of escape" or "escape response." A rat which learns to press a bar to turn off an electric shock is performing an instrumental act of escape which it learns as a result of escape training (9, p. 56).

The disagreeable, distressing, and noxious stimuli of withdrawal symptoms may be terminated by an intake of morphine. Any response, therefore, which results in a morphine-

* Received August 15, 1958, from the University of Oklahoma, College of Pharmacy, Norman.

Based, in part, upon a Ph D. dissertation submitted to the Graduate College, University of Oklahoma, by the senior author.

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The authors are indebted to Dr. Irene Mackintosh for many valuable suggestions made during the preparation of this article.

intake will be reinforced by termination of the withdrawal distress and tend to be repeated.

This experiment attempts to manipulate the withdrawal intensity in groups of rats during the course of escape training. Differing degrees of withdrawal intensity should be reflected in different degrees of learning the instrumental act of escape. In this experiment, the escape response is drinking a morphine solution.

PROCEDURE

The experiment was conducted in a temperature-controlled room (72° F.). Thirty-nine female Sprague-Dawley rats weighing between 185 and 250 Gm. served as subjects. To establish motivation (withdrawal symptoms), daily intraperitoneal injections of morphine hydrochloride were started at 20 mg./Kg. and increased by equal increments to 160 mg./Kg. on the 29th day. Nine rats died during this period.

The remaining 30 morphine-tolerant, morphine-dependent rats were randomly assigned, 10 to group I and 20 to group II. Escape training cycles were then begun. In man, withdrawal distress reaches a peak forty-eight to ninety-six hours after the last opiate intake (12). Stanton (13) gives graphs which indicate that rats injected for four weeks show a peak of struggling when restrained during withdrawal which is similar to this time interval. Because of this, the escape training cycles were made three days long. Drinking the morphine solution reduced the current withdrawal symptoms and "re-set" the animal for a return of the symptoms three days later. One complete training trial (or cycle) consisted of twenty-four hours with no liquids (*O*), twenty-four hours with an 0.5 mg./cc. morphine hydrochloride solution (*M*), and twenty-four hours with tap water (*W*). Training proceeded as in this sequence (illustrating three trials and covering nine days): *O, M, W, O, M, W, O, M, W*, etc. The rats were individually caged and the solutions presented in 100-ml. graduated drinking tubes randomly located in the right or left positions on the front of the cage.

To test drug-seeking behavior, five choice tests were made in which two drinking tubes, one containing water and the other morphine solution, were presented simultaneously for a twenty-four hour period. Untrained rats refuse to drink the *M* solution, averaging 0.9 cc. under these conditions. For *M* consumption over 10 cc., the amount was read directly from the tube graduations. Amounts of *M* less than 10 cc. were measured by refilling the tube using a hypodermic syringe. Between the third and fourth, and between the fourth and fifth choice tests, the rats were removed from their individual, wire bottomed cages and placed in one large, straw litter-bottomed cage.

In previous experiments (3) it had been found that rats which drank less than 35 cc. of *M* on half or more of their training trials also drank very little (mean was 1.8 cc.) on their choice tests. In contrast, rats which drank more than this level averaged 13.6 cc. of *M* on their choice tests. This is consistent with the experimental design. A certain minimal morphine-intake is necessary to maintain tolerance, physical dependence, and thus, withdrawal symptoms from trial to trial during the training period.

Because of the small groups in this experiment, it was felt that these "minimal drinkers" could seriously reduce the precision of the experiment. Accordingly, a somewhat conservative rejection criterion was established. Rats which drank less than 20 cc. on half or more of their training trials continued to receive their assigned treatment and choice scores were obtained from them; but their scores were eliminated from statistical consideration when computing the main effects of the experiment.

All rats received the escape training treatment. In addition, the 20 rats in group II received three small morphine injections (2 mg. ea.) per cycle. Two injections were given on day (*O*) six and one-half and one and one-half hours before the beginning of day (*M*). The third injection was given on day (*W*) eight hours after the end of day (*M*).

After the first choice test, group II was randomly divided into two equal subgroups: IIA and IIB Subgroup IIA continued the injection schedule given above for Group II. In IIB, the injection one and one-half hours before day (*M*) was eliminated. A new injection for IIB was then added one hour after the beginning of day (*O*). This new injection time was about midway between the two remaining original injection times. IIA and IIB continued these schedules to the end of escape training.

Due to a nonnormal distribution of scores, a nonparametric statistical technique, the Kruskal-Wallis one-way analysis of variance by ranks (11), was used.

RESULTS

Two rats (one each in IIA and IIB) died prior to the first choice test. Five rats (two in group I, one in group IIA, and two in group IIB) drank less than 20 cc. on half or more of their training trials and were eliminated by the rejection criterion. As a result, the final number of subjects was 8 in group I, 8 in group IIA, and 7 in group IIB.

The animals eliminated by the rejection criterion drank significantly less *M* than the nonrejected rats on all choice tests (p of 0.005, 0.01, 0.005, 0.01, and 0.01). The mean *M* intake for all choice tests for the eliminated rats was 2.6 cc.; that for the nonrejected rats was 12.3 cc. The mean oral intake of morphine on the training trials by the nonrejected rats was approximately 120 mg /Kg. per rat (Fig. 1).

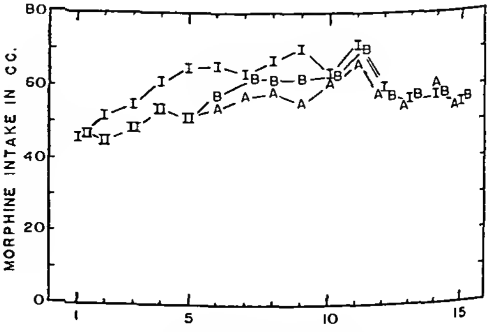

Fig. 1.—Mean consumption of morphine solution on 15 training trials.

Although IIA and IIB received small injections, neither the total morphine intake, nor the oral intake of *M* on the training trials (Fig. I) differed significantly among the groups. Figure 4 gives the mean cc. intake of tap water on the choice tests. One difference, II *vs.* I for the second choice test, was significant at 0.05. Total liquid intake (tap water plus *M*) is given in Table I. Group differences in total liquid intake are not significant. Whether considered by groups or by combinations of groups, the following comparisons are nonsignificant; group differences in *M* intake on individual training trials, total morphine intake before each choice test, and total morphine intake for the experiment.

Figure 2 shows the mean oral intake of *M* by groups on choice tests (not training trials). The *M* intakes are significantly different when the combined injected groups are compared with the noninjected group (II *vs.* I). The significances of these differences on the first three choice tests are, respectively, 0.05, 0.005, and 0.05. This comparison approached significance (0.08) on the first abstinence choice test. No individual group comparisons were found to be significant; however, IIB showed the greatest mean morphine intake on all tests (Fig. 2), and had the only two rats which showed a highly pronounced relapse (Fig. 3). On the first abstinence test, one animal consumed 74 cc. of *M* (equivalent to 160 mg./Kg.), and another the equivalent of 98 mg./Kg. Even after twenty-nine more days of abstinence, the latter rat consumed 71 mg./Kg.

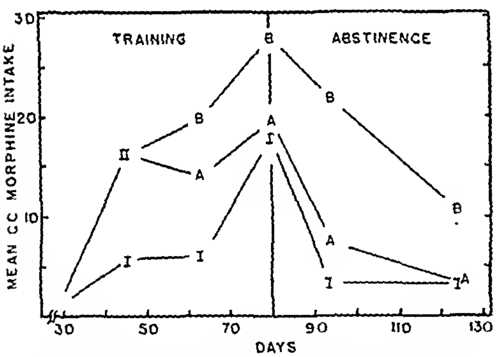

Fig 2.—Mean consumption of morphine solution on 5 choice tests.

DISCUSSION

Consistent with previous findings (3), additional evidence indicates thirst is not an important factor in the procedure of this experiment. As morphine consumption increases, water intake decreases so that the total liquid intake remains essentially constant (Figs. 2, 4; Table I). There are no significant differences among the three groups in total liquid intake, nor for injected *vs.* noninjected groups (II *vs.* I).

The decrease in total liquid intake on choice tests four and five (during abstinence), as shown in Table I, reflects a change in availability of liquids. Rats, when put on a schedule which prohibits liquid intake one day out of three, increase their consumption on those days when liquids are available. Then, when transferred to an *ad libitum* drinking situation, this increase due to a restricted intake tends to disappear so that consumption approaches normal levels.

TABLE I.—MEAN CC. TOTAL LIQUID INTAKE BY GROUPS

Choice Test	I	IIA	IIB
1	65.1	65.8	67 9
2	66.5	58.8	60.4
3	76.5	57.7	70 8
4	43.0	44.3	47 2
5	49.8	57.1	50.0

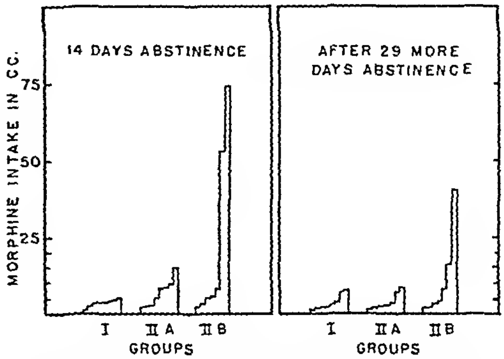

Fig. 3.—Graphic representation of morphine intake by individual rats on choice tests after periods of abstinence from morphine.

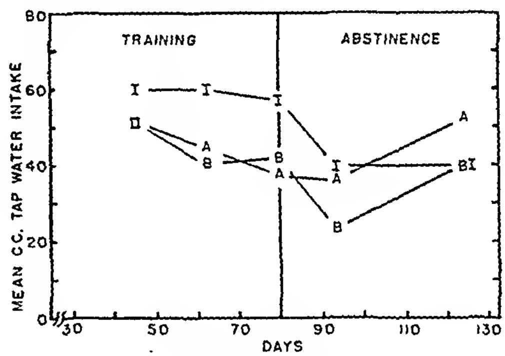

Fig. 4.—Mean cc. intake of tap water on the five choice tests.

Because tube positions were randomly determined during training and for choice tests, place learning cannot account for the results of this experiment. Neither are the results due to novel stimuli (3), nor can they be considered as artifacts produced by use of the rejection criterion. A comparison of the M intake of the injected groups with that of the noninjected group for all animals from which choice scores were obtained ($N = 28$), still gives significant differences of 0.01 and 0.025 for the second and third choice tests, respectively. Total morphine intake does not differ significantly for the same comparison. Also, the significant differences between the rejected and nonrejected rats indicate they may be justifiably considered as samples from different populations. It may be concluded then, that use of the criterion does not alter the general picture but, rather, provides a more precise measure of the experimental effects.

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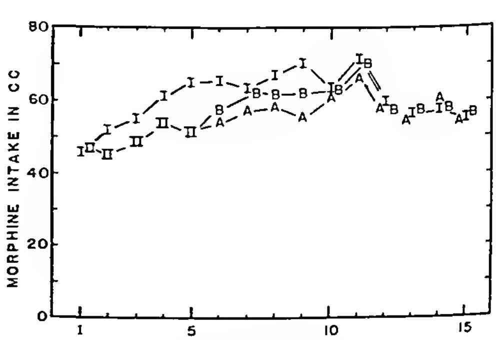

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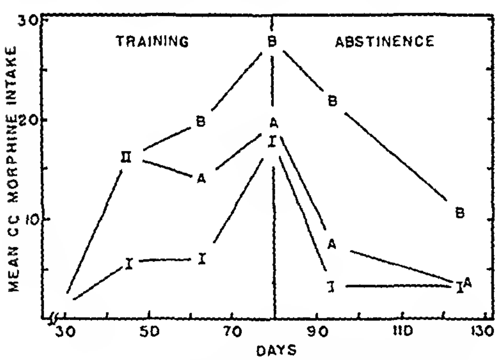

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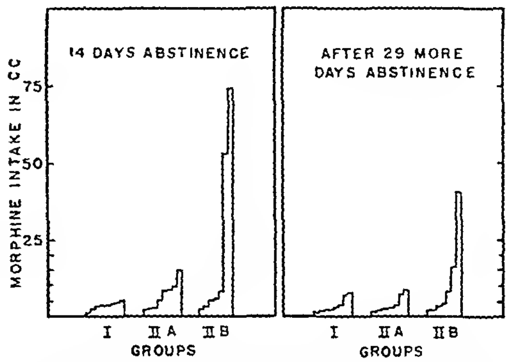

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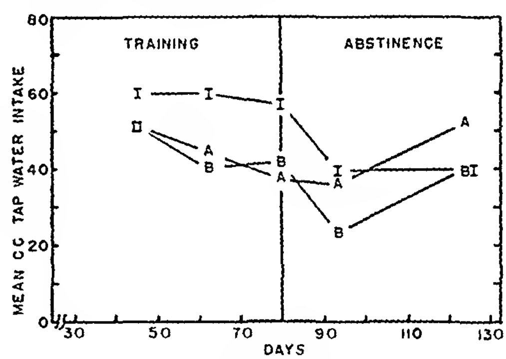

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Before the first choice test, the effect of the injections given to Group II could not be predicted with any certainty due to the complexity of the si tion. There is an intimate relationship

drawal symptoms, physical dependence, and tolerance to the extent that it is possible to consider them as only different manifestations of one fundamental process (12). The question of effect of the injections was answered by the first choice test. The injected group was learning the response better than the noninjected group. A possible explanation of the results of this first choice test was that the injections acted to better maintain dependence and tolerance during the forty-eight hour interval between opportunities to drink the *M* solution Because of this explanation, a new injection schedule for group IIB was established This hypothesis, that dependence and tolerance were not well maintained during the inter-trial period under the noninjection procedure, is consistent with the final results of the experiment. The 15 training trials used in this experiment did not noticeably increase *M* intake on the choice tests over that obtained with only 10 training trials used in previous experiments (3). An inefficiency in later training trials would be expected if dependence and tolerance were decreasing during the course of the experiment. Although all rats received increasing doses of morphine up to 160 mg /Kg. before the experiment and averaged more than 100 mg./Kg. of morphine per training trial, this apparently was insufficient to maintain a high level of tolerance and dependence through the later training trials.

If the injections given group II did, in fact, help maintain inter-trial dependence, then the new injection schedule for IIB could be expected to maintain inter-trial dependence even better since the injections were more evenly spaced for the inter-trial period. Maximum time without morphine on the original injection schedule was thirty-four hours. This was reduced to seventeen hours on the new schedule. The prediction of outcome at the time IIB was established was that group I would be low, IIA intermediate, and IIB highest in *M* intake on the choice tests. The fact that this order was obtained on all subsequent choice tests (Fig. 2); the form of the distribution of scores on the relapse tests (Fig. 3); and the two substantial relapses in IIB, all lend some support to the inter-trial maintenance-of-dependence hypothesis, although statistical significance could not be demonstrated for the differences between IIA and IIB.

The central finding of this experiment is that the injections did bring about significant increases in drug-directed behavior. Since differences in amount of morphine received cannot account for these increases, an explanation on some other basis must be advanced. It seems possible that the important difference between the injected and noninjected rats was a difference in their physiological state at the time morphine was presented. Some evidence on the interaction between physiological state and morphine intake is available in statements made by opiate addicts. They prefer to allow some withdrawal symptoms to develop before taking morphine. They try to explain this behavior by comparing it to hunger, "thus, 'eating a steak may always be enjoyable, but more so if one is hungry' " (12, p. 35). It appears possible, therefore, that the importance of the injections in the development of drug-seeking behavior in this experiment is due to the fact that they helped to better maintain and preserve, from trial to trial, the physiological state necessary for reinforcing the escape response, i.e., withdrawal symptoms. This explanation is consistent with the success of this and previous experiments (3) in producing drug-directed behavior, since the design of these experiments is based upon escape training and assumes the importance of withdrawal symptoms in reinforcing the escape response.

The most important conclusion to be drawn from this experiment is that the commonly supposed "inherent rewarding effect" of morphine is not sufficient to account for the results since differential effects were produced among the groups without significant differences in morphine intake. There were no significant differences in *M* intake on training trials, nor in total amount of morphine received before each choice test, nor in total morphine intake for the entire experiment. To hold that the results of this experiment depend on an "inherent rewarding effect" of morphine seems inconsistent with the fact that equivalent rewards for the three groups (since the amounts of "inherently rewarding morphine" did not differ significantly) produced unequal effects among the groups. The rats in control groups of previous experiments (3) also received at least as much morphine as the rats in their associated experimental groups but did not develop drug-seeking behavior. The difference produced in the behavior of these rats seems more closely related to the conditions under which morphine is received than it is to the amount of morphine received. This difference is similar to the difference in the behavior of man produced under different conditions of morphine intake (illegal users *vs.* hospital patients). It appears that for rats, as for man, the conditions under which morphine is received is a much more important factor in producing drug-directed behavior than the amount of morphine received. Thus, any explanation of drug-directed behavior in terms of some inherent rewarding properties of morphine seems insufficient to fully account for the drug-directed behavior of either rats or man.

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The Spectrophotometric Assay for Chlortetracycline[1] HCl and Tetracycline[1] HCl in Pharmaceuticals*

By FORTUNATO S. CHICCARELLI, MASON H. WOOLFORD, Jr., and MAURICE E. AVERY

Chlortetracycline heated under acid conditions forms anhydrochlortetracycline while tetracycline under the same conditions forms anhydrotetracycline. Anhydrochlortetracycline and anhydrotetracycline have similar spectrophotometric properties which make a distinction unreliable. Specific assays have been developed for chlortetracycline and tetracycline based on the formation of anhydro compounds and the fact that chlortetracycline heated at pH 7.5 is cleaved to isochlortetracycline while tetracycline under the same conditions is relatively stable.

Since its introduction in 1948, chlortetracycline has been an important therapeutic agent in medical practice, while tetracycline (1, 2), the most recent of the tetracyclines, has become the most widely used broad spectrum antibiotic. Certification still requires the performance of a microbiological assay but this report will deal with the chemical assays currently in use by the Quality Control Section of Lederle Laboratories for the rapid and efficient control of production items.

Many of the fundamental chemical and physical properties of chlortetracycline serving as the basis for chemical assay have been described previously. Among these are paper strip chromatography (3, 4), polarography (5), nonaqueous titrimetry (6), fluorescence (7–9), and chemical color development (10–14). The assay of this compound in mixtures has also been proposed (17–19).

The chlortetracycline in the sample taken for analysis is brought into aqueous solution to a final concentration of approximately 100 µg./ml. Hydrochloric acid is added to one aliquot of the solution and the mixture is heated forming colored anhydrochlortetracycline which has an absorption maximum at 445 mµ. A second aliquot is heated at pH 7.5 and the chlortetracycline is cleaved to isochlortetracycline. Subsequent acid treatment does not rearrange the isochlortetracycline into anhydrochlortetracycline; thus the second aliquot can be used as the blank when calculating the chlortetracycline present.

Chemical methods suggested for chlortetracycline and oxytetracycline (10–16) for the most part are applicable to tetracycline because the three are related structurally. Paper strip chromatography (3, 4), polarography (5), and nonaqueous titrimetry (6) can also be adapted. Since tetracycline does not fluoresce under basic conditions, a fluorescence type assay similar to the one used for chlortetracycline (7, 9) cannot be adapted. Under basic conditions tetracycline solutions have a relatively stable color exhibiting a maximum at 380 mµ (20). The assay of mixtures has also been proposed (17–19).

The tetracycline in the sample taken for analysis is brought into aqueous solution to a final concentration of approximately 200 µg./ml. Two separate 5.0-ml. aliquots are heated with pH 7.5 buffer. The chlortetracycline is cleaved to isochlortetracycline while the tetracycline remains unaltered. One of the solutions is removed and cooled. After the addition of acid this solution is used as the blank. Acid is added to the second solution and heating is continued to form anhydrotetracycline. The second solution can be used as the assay sample. Blank and sample are measured at 434 mµ.

PHARMACEUTICALS CONTAINING CHLORTETRACYCLINE HCl

Specific Assay for Chlortetracycline

The spectrophotometric assay described in this report is a modification of the method of Levine, *et al.* (7), which appears in a volume by Grove and Randall (14), and is carried out according to the following scheme:

$$\begin{matrix}\text{chlortetracycline} \\ \text{(tetracycline)}^2\end{matrix} \xrightarrow[\text{HCl}]{\text{heat}} \begin{matrix}\text{anhydrochlortetracycline} \\ \text{(anhydrotetracycline)}\end{matrix}$$

Anhydrochlortetracycline (21) has an absorption maximum at 445 mµ while anhydrotetracycline has absorption maximum at 434 mµ. It is apparent that the above approach does not compensate for the presence of small concentrations of tetracycline

* Received August 25, 1958, from the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

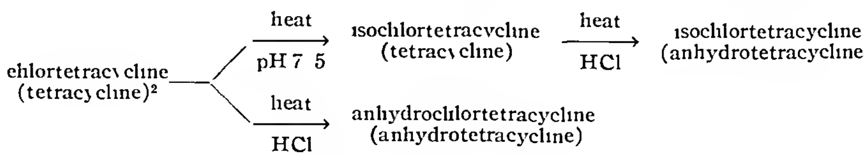
[1] Trademark of Lederle Laboratories Division, American [illegible] racycline is Aureomycin and for [illegible]

[illegible] nowledge with gratitude the kind [illegible] the completion of this work, and [illegible] D. Piersma and Mr. P. Van Gieson for numerous suggestions.

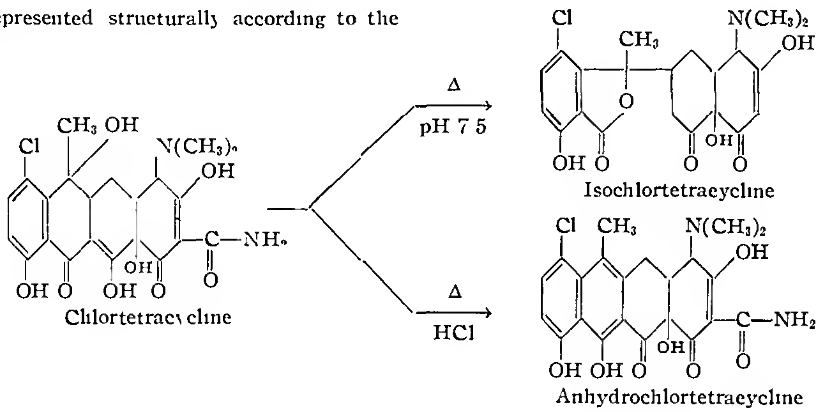
[2] Parentheses are used to show what would happen to low concentrations of tetracycline if present in chlortetracycline samples being assayed.

in chlortetracycline, which would be additive, and does not guarantee color production as coming from chlortetracycline only The procedure of Hiscox (10) also uses this fundamental reaction but embodies measurements from different parts of the spectrum, and the assay is not as specific for chlortetracycline as that of Levine, *et al*

A method previously described by Chiccarelli, *et al* (22), eliminates these objections and is performed according to the following scheme



This is represented structurally according to the following



Oxytetracycline will not interfere with the above, since colorless apooxytetracyclines (23) are formed Tetracycline because of its stability under basic conditions will form anhydrotetracycline both in the blank and sample Since oxytetracycline is converted to colorless compounds in the blank and sample solutions and since tetracycline under the same conditions forms a mutually compensating color, the difference in absorbance between the blank and sample solution will result only from chlortetracycline

Curve A of Fig 1 is the absorption spectrum of chlortetracycline in 0 1 N hydrochloric acid, curve B is the absorption spectrum of isochlortetracycline under the conditions of the assay, and curve C is the absorption spectrum of anhydrochlortetracycline under the conditions of the assay

Table I lists the concentration curve within the limits of the assay

Table II lists time as a variable in the preparation of blank and sample solution At the end of each time interval, hydrochloric acid was added and heating was continued in each case for a total of seven minutes Thus on line 2 the solution was heated for two minutes at pH 7 5 then the acid was added and heating continued for five minutes more It may be seen from Table II that seven minutes is optimum heating time for both the sample and the blank solution

The assay is performed in the presence of sodium bisulfite which has a stabilizing effect on the back ground color during the pH 7 5 treatment

Procedure for Application of This Assay to Pharmaceutical Products

Reagents.—Buffer solution pH 7 5—178 Gm of anhydrous K_2HPO_4 and 22 Gm of anhydrous KH_2PO_4 per liter of water, filter before using, sodium bisulfite—10 Gm per 100 ml of water (freshly prepared), and hydrochloric Acid—5 N

Procedure.—Final dilutions before acid and heat treatment should contain about 100 µg of chlortetra

TABLE I

Concentration µg /ml	Corrected Absorbance[a]	Absorptivity at 445 mµ
10 0	0 131	131 0
20 0	0 260	130 0
30 0	0 393	131 0
40 0	0 528	132 0
		Average 131 0

[a] Absorbance of sample solution minus absorbance of blank solution

TABLE II

Blank[a] Time min [b]	Blank[a] Absorbance at 445 mµ	Sample[a] Time min [c]	Sample[a] Absorbance at 445 mµ
1	0 070	1	0 017
2	0 025	2	0 050
3	0 010	3	0 088
4	0 006	4	0 117
5	0 004	5	0 127
6	0 004	6	0 134
7	0 004	7	0 134
		8	0 134
		9	0 131
		10	0 130
		11	0 125
		12	0 122

[a] Chlortetracycline standard initial concentration 10 µg /ml
[b] This column shows the time during which the solution was heated at pH 7 5
[c] This represents the heat treatment while the solution was acidic

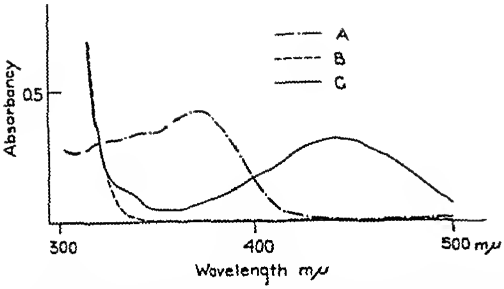


Fig. 1—Absorption spectrum from 300 mμ to 500 mμ of chlortetracycline, at 20 μg./ml. A in 0.1 *N* HCl; B under conditions of blank preparation; and C under conditions of sample preparation.

cycline per ml. See method of sample preparation.

Pipet a 10-ml. portion of the final dilution into each of two 50-ml. volumetric flasks. One of these will be the blank and the other the sample. To the sample add in this order: 12 ml. of 5 *N* HCl, 15 ml. of pH 7.5 buffer, and 2 ml. of bisulfite solution. Suspend in a boiling water bath for exactly seven minutes. Swirl occasionally.

To the blank add 15 ml. of pH 7.5 buffer solution and 2 ml. of sodium bisulfite solution. Suspend in a boiling water bath for five minutes with occasional swirling. After exactly five minutes add 12 ml. of 5 *N* HCl and heat for an additional two minutes.

After the completion of the heat treatment immediately cool both the blank and sample under tap water. Make to volume with water and mix well. Determine the absorbance at 445 mμ against water using a suitable spectrophotometer.

$$\frac{(A_{445}\text{ sample} - A_{445}\text{ blank})}{131.0^{3}} \times \frac{\text{dilution factor}}{\text{wt. of sample}} = \%\text{ chlortetracycline HCl}$$

Sample Preparation

Capsules, Tablets, Powders, and Pharyngets[4].—Take an amount of sample equivalent to about 100 mg. of chlortetracycline. Transfer to a liter volumetric flask and add about 200 ml. of water. Mix until solution is complete. Make to mark with water. If filtration is necessary, use Whatman No. 42 paper. (Discard the first 10 ml. before taking an aliquot for assay.) Follow procedure.

Syrup, Pediatric Drops, and Cream.—Since calcium chlortetracycline is used in this type product, it will be necessary to add a small amount of hydrochloric acid in order to bring the chlortetracycline into solution. Transfer by weight or with a washout pipet a sample equivalent to about 100 mg. of chlortetracycline to a liter volumetric flask. Add 200 ml. of water and a small amount of dilute hydrochloric acid. Shake until solution is complete. Make to mark with water and mix well. Filter if necessary. Follow procedure.

Ointments.—Transfer a sample weight equivalent to about 100 mg. of chlortetracycline to a liter flask. Add exactly 100 ml. of CCl_4 and about 100 ml. of H_2O. Shake until sample is completely dispersed. Make to mark with water and mix well. Follow procedure. Do not consider the CCl_4 added as part of the dilution in the calculation. This fraction of the dilution will be 9 rather than 10.

Intravenous.—Weigh accurately about 150 mg. of powder. Transfer with the aid of 100 ml. of 0.1 *N* HCl to a liter flask. Make to mark with water and mix well. Follow procedure.

Spersoids[5] Dispersable Powder.—Weigh accurately about 3.0 Gm. of Spersoids. Transfer to a 500-ml. volumetric flask with about 100 ml. of water. Add 10 ml. of 1 *N* HCl and mix until all the lumps are dispersed. Make to mark with water and mix well. Filter through an S-1 filter pad on a Büchner funnel, discarding the first 100 ml. Follow procedure.

Troches.—Chlortetracycline troches contain a dye which interferes with the general spectrophotometric assay. In order to separate the dye from the chlortetracycline, a special modification of the spectrophotometric assay was developed. The assay is carried out in the following manner: Pulverize about five troches, accurately weigh the equivalent of about 30 mg. of chlortetracycline and transfer to a 250-ml. volumetric flask. Add about 100 ml. of water and mix until solution is complete. Make to mark with water and mix well. Using Whatman No. 42 filter paper, filter a portion of the sample. Discard the first ten ml. before taking a sample for assay. Pipet a 25-ml. aliquot into two glass-stoppered 50 ml. centrifuge tubes each containing 200 mg. of Magnesol magnesium trisilicate (Westvaco). The Magnesol should be slurried with water, filtered and dried before use. Shake for five minutes. Centrifuge. Decant the liquid carefully. Now add 15 ml. of water and mix well for three minutes. Centrifuge and decant the liquid.

Transfer each of the Magnesol and chlortetracycline complex samples to separate 100-ml. volumetric flasks with about 25 ml. of water. One of these will be the blank and the other will be the sample. To the sample add in this order: 24 ml. of 5 *N* HCl, 30 ml. of pH 7.5 buffer, and 4 ml. of bisulfite solution. Suspend in a boiling water bath for exactly seven minutes. Swirl occasionally.

To the blank add 30 ml. of pH 7.5 buffer and 4 ml. of bisulfite solution. Suspend in a boiling water bath for five minutes. This solution must be swirled constantly. After exactly five minutes have elapsed add 24 ml. of 5 *N* HCl and heat for an additional two minutes.

After completion of the heat treatment, immediately cool both the blank and the sample under tap water. Make to volume with water and mix well. Centrifuge a portion of the blank and sample. Determine the absorbance at 445 mμ with water in the reference cuvette. The absorptivity of chlortetracycline HCl under the above conditions of assay is 122.0. This value should be used rather than the one given in the general method of assay when calculating the amount of chlortetracycline per troche.

If available, a standard should be determined with each sample. This can serve as a check on the conditions of assay.

[3] Absorptivity of chlortetracycline HCl under assay conditions. A standard should be determined to ascertain the absorptivity under the particular conditions in each laboratory.

[4] Trademark of American Cyanamid Co.

[5] Trademark of American Cyanamid Co.

PHARMACEUTICALS CONTAINING TETRACYCLINE HCl

Specific Assay for Tetracycline

Tetracycline when heated under acid conditions forms anhydrotetracycline (21). An assay based on this reaction appears in a volume by Grove and Randall (14) and is carried out according to the following scheme (15):

$$\begin{matrix}\text{tetracycline} \\ (\text{chlortetracycline})^{6}\end{matrix} \xrightarrow[\text{HCl}]{\text{heat}} \begin{matrix}\text{anhydrotetracycline} \\ (\text{anhydrochlortetracycline})\end{matrix}$$

Anhydrotetracycline has a maximum at 434 mμ while anhydrochlortetracycline has a maximum at 445 mμ. The above approach does not distinguish between tetracycline and chlortetracycline and does not compensate for the presence of small concentrations of chlortetracycline in tetracycline.

A modification has been made of the method of Grove and Randall whereby the assay is made specific for tetracycline according to the following:

Curve A of Fig. 2 is the absorption spectrum of tetracycline in 0.1 N HCl; curve B is the absorption spectrum of tetracycline under the conditions of blank preparation; and curve C is the absorption spectrum of anhydrotetracycline under the conditions of the assay. Table III lists the concentration curve within the limits of the assay.

During the preparation of the sample, the solution is placed in a boiling water bath for three minutes at pH 7.5. Possible chlortetracycline interference is thus eliminated (7). Table IV lists time as a variable in the preparation of the sample solution. Each solution was heated at pH 7.5 for three minutes. Acid was added. After each time interval the solution was cooled and made up to volume. Five minutes is the optimum heating time for the sample solution after the addition of acid.

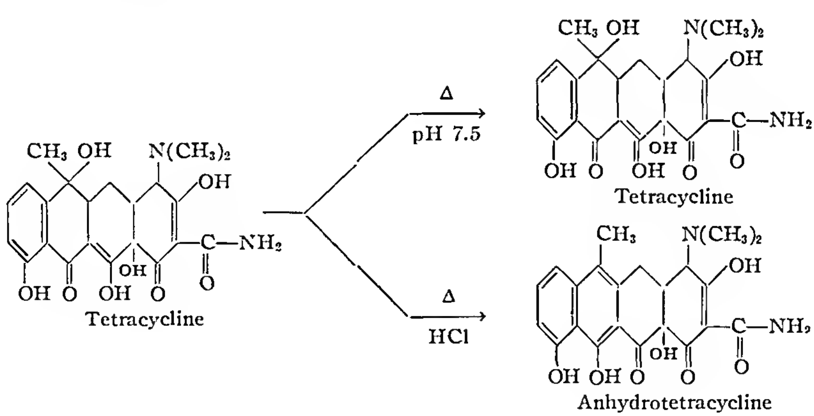
The assay is performed in the presence of sodium bisulfite which has a stabilizing effect on the background color.

$$\begin{matrix}\text{tetracycline} \\ (\text{chlortetracycline})^{6}\end{matrix} \xrightarrow[\text{pH 7 5}]{} \begin{matrix}\text{tetracycline} \\ (\text{isochlortetracycline})\end{matrix} \xrightarrow[\text{divided in half}]{\text{sample}}$$

$$\xrightarrow[\text{HCl}]{\text{after cooling}} \begin{matrix}\text{tetracycline} \\ (\text{isochlortetracycline})\end{matrix}$$

$$\xrightarrow[\text{HCl}]{} \begin{matrix}\text{anhydrotetracycline} \\ (\text{isochlortetracycline})\end{matrix}$$

This is represented structurally:



Tetracycline when heated at pH 7.5 is relatively stable and can be acid rearranged to anhydrotetracycline. Chlortetracycline, when heated at pH 7.5, forms isochlortetracycline which cannot be acid rearranged to anhydrochlortetracycline. Oxytetracycline, more stable under basic conditions, forms apooxytetracyclines (23) when heated with acid. Isochlortetracycline and apooxytetracyclines are colorless, so that any difference in absorbance at 434 mμ between blank and sample will result only from tetracycline.

Procedure for Application of This Assay to Pharmaceutical Products

Reagents.—Buffer solution pH 7.5.—178 Gm. of anhydrous K_2HPO_4 and 22 Gm. of anhydrous KH_2PO_4 per liter of water, filter before using; sodium bisulfite—10 Gm. per 100 ml. of water, freshly prepared; and hydrochloric acid—5 N.

Procedure.—Final dilutions before acid and heat treatment should contain about 200 μg. of tetracycline per ml. See method of sample preparation.

Pipet a 5.0-ml. portion of the final dilution into each of two 50-ml. volumetric flasks. To both flasks add 1 ml. of bisulfite solution and 10 ml. of

[6] Parentheses are used to show what would happen to low concentrations of chlortetracycline if present.

TABLE III.

Concentration µg./ml.	Corrected Absorbance[a]	Absorptivity at 434 mµ
5 0	0.081	162.0
10 0	0.161	161.0
15.0	0.243	162.0
20.0	0.320	160.0
25.0	0.402	160.0
		Average 161.0

[a] Absorbance of sample solution minus absorbance of blank solution.

TABLE IV.

Time, min.[a]	Corrected Absorbance at 434 mµ[b]
0	0.056
1	0 265
2	0.310
3	0.320
4	0 323
5	0.323
6	0.323

[a] This represents the heat treatment while the solution was acidic
[b] Tetracycline standard initial concentration: 20 µg./ml.

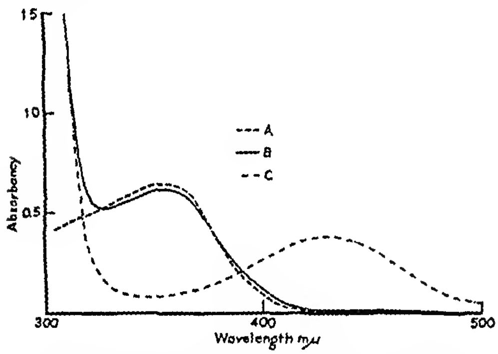


Fig. 2.—Absorption spectrum from 300 mµ to 500 mµ of tetracycline at 20 µg./ml. A in 0.1 *N* HCl; B under conditions of blank preparation; and C under conditions of sample preparation.

buffer solution. Place in a boiling water bath for exactly three minutes. Swirl occasionally during this period. After the three minutes have elapsed, remove one of the flasks and cool. This will serve as the blank.

To the remaining flask, add 6 ml. of 5 *N* HCl and heat for an additional five minutes with occasional swirling. This will be the sample.

After the blank has cooled, add enough water to give a total volume of about 40 ml. and add 6 ml. of 5 *N* HCl to the flask. Make to mark with water and mix well. When the sample has cooled, make to mark with water and mix well.

Determine the absorbance of the blank and sample at 434 mµ as soon as possible using water in the reference cuvette.

$$\frac{(A_{434}\text{ sample} - A_{434}\text{ blank})}{161.0^{7}} \times \frac{\text{dilution factor}}{\text{wt. of sample}} = \%\text{ tetracycline HCl}$$

[7] Absorptivity of tetracycline HCl under assay conditions A standard should be determined to ascertain the absorptivity under the particular conditions in each laboratory.

Sample Preparation

Capsules, Powders, Tablets, and Pharyngets[4].—Take an amount of sample equivalent to about 100 mg. of tetracycline. Transfer to a 500-ml. volumetric flask and add about 200 ml. of water. Mix until solution is complete and make to mark with water. If filtration is necessary use Whatman No. 42 paper. Discard the first 10 ml. before taking an aliquot for assay. Follow procedure.

Suspensions, Syrups, and Pediatric Drops.—Since tetracycline neutral is used in this type product, it will be necessary to add a small amount of hydrochloric acid in order to bring the tetracycline into solution. Transfer by weight or with a washout pipet a sample equivalent to about 100 mg. of tetracycline to a 500-ml. volumetric flask. Add 200 ml. of water and a small amount of dilute hydrochloric acid. Shake until solution is complete. Make to mark with water. Filter if necessary. Follow procedure

Ointments.—Transfer a sample weight equivalent to about 50 mg. of tetracycline to a 250-ml. volumetric flask. Add exactly 50 ml. of CCl_4 and about 50 ml. of H_2O. Shake until sample is completely dispersed. Make to mark with water and mix. Follow the procedure. Do not consider the CCl_4 added as part of the dilution in the calculation.

Spersoids[5] Dispersible Powder.—Transfer a 3-Gm. sample (50 mg. of tetracycline) with the aid of water to a 250-ml. volumetric flask. Add 10 ml. of 1 *N* HCl and mix until all lumps are dispersed. Make to mark with water and mix well. Filter through an S-1 filter pad on a Büchner funnel, discarding the first 75 ml. Follow procedure.

Oil Suspension.—Using a washout pipet, transfer a 5.0-ml. sample (50 mg. of tetracycline) to a 250-ml. volumetric flask. Wash the pipet with acetone (25 ml.). Mix. Add slowly and with constant swirling, 20 ml. of 1 *N* HCl and about 100 ml. of water. When the oil has coagulated, dilute to mark with water and mix. Filter through Whatman No. 42 paper, discarding the first 10 ml. Follow procedure. Do not consider the oil as part of the dilution in the calculation.

Troches.—Use the isolation and purification technique described under chlortetracycline troches. After this, transfer the Magnesol magnesium trisilicate residues containing tetracycline to separate 100-ml. volumetric flasks with about 25 ml. of water. One of these will be the blank and the other the sample.

Now add 1 ml. of bisulfite solution and 10 ml. of buffer solution to each flask. Suspend in a boiling water bath for four minutes. Swirl occasionally. After the four minutes have elapsed, remove one flask and add 6 ml. of 5 *N* HCl to the other. Cool the one removed, make to about 90 ml. with water, add 6 ml. of 5 *N* HCl then dilute to mark and mix well. This will be the blank.

After adding 6 ml. of 5 *N* HCl to the flask in the boiling water bath, heat for an additional five minutes. Cool and make to mark with water. This will be the sample.

Centrifuge a portion of the blank and sample. Determine the absorbance at 434 mµ with water in the reference cuvette. The absorptivity of tetracycline HCl under the above conditions of assay is 155.0. This value should be used rather than the

one given in the general method of assay, when calculating the amount of tetracycline per troche

If available, a standard should be determined with each sample This can serve as a check on the conditions of assay

SUMMARY

1 Specific spectrophotometric assays have been developed for tetracycline and chlortetracycline

2 Procedures are described in which the assays may be used for various types of pharmaceutical products

3 An isolation technique has been developed that may be adapted to other problems

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Polarography of Some Phenylmercury Amide and Imide Compounds*

By SISTER M. LAWRENCE O'DONNELL and CORNELIUS W. KREKE

Several amide and imide compounds of phenylmercury were prepared and their stability studied polarographically under different conditions. The half-wave potentials of these compounds were found to be identical, within experimental error, to the decomposition potential of phenylmercuric hydroxide, suggesting a ready hydrolysis in each case. Other characteristics of the polarograms were also compared. Polarograms of mixtures of the coordinating compounds with phenylmercuric hydroxide in molar ratios greater than 1:1 were obtained. An increase in the half-wave potentials to greater negative values in the case of the amides of higher molecular weight and also succinimide indicates a greater binding of these compounds with phenylmercury as contrasted with the amides of lower molecular weight which did not shift the half-wave potentials under the conditions employed.

IN A RECENT PUBLICATION by Smalt, *et al* (1), several phenylmercury compounds were studied as biological inhibitors with oxidative enzymes as test systems The authors found that with the exception of phenylmercuric succinimide and phenylmercuric phthalimide all the compounds studied were quantitatively similar to phenylmercuric hydroxide, and concluded that the compounds dissociated readily in water presumably acting on the enzymes as the free phenylmercuric ion

The mode of action of the organic mercurials is assumed to involve a specific reaction with sulfhydryl groups of the proteins of the cell (2) Still greater specificity is also postulated by Hughes (2), selective binding sites among the sulfhydryl groups resulting in differences in biological action as diuretics, germicides, fungicides, antiseptics, and antisyphilitics The situation is complicated, however, since some enzymes which are normally considered nonsulfhydryl dependent are also inhibited by the mercurials (3, 4) suggesting that in these cases the phenylmercuric ion or the undissociated molecule reacts nonspecifically with other essential groups

Since the phthalimide compound and to a lesser extent the succinimide compound were not as effective as phenylmercury hydroxide in the depression of enzyme activity, the inference can be drawn that they are less readily hydrolyzed,

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an inference which seems strange in view of the fact that phenylmercury amide compounds were among the most effective as inhibitors

In order to test this hypothesis, several phenylmercury amide and imide compounds were prepared and tested polarographically for their stability in water solution Comparisons were made with the parent compound, phenylmercuric hydroxide, in the magnitude of the half-wave potentials

EXPERIMENTAL

Preparation of Compounds—Equimolar mixtures (0 01 moles) of the phenylmercuric hydroxide (Berk & Co), recrystallized from water and the amide or imide (Eastman) compounds were placed in a flask and 100 ml of water added This mixture was refluxed for thirty minutes and the solid precipitate filtered off and recrystallized from water or an alcohol-water mixture The compounds were dried in the vacuum desiccator and analyzed for mercury by the method of Pierce (5) The compounds prepared are given in Table I along with their analyses and melting points

TABLE I—MELTING POINT AND ANALYSIS OF SOME PHENYLMERCURY COMPOUNDS

Compounds	Mercury % Calcd	Mercury % Found	Melting Point Uncorrected °C
PM hydroxide[a]			224–227
PM acetate[a]			147–150
PM acetamide[a]			160–162
PM propionamide	57 35	57 54–56 09	95–96
PM butyramide	55 14	57 79–55 54	66–67
PM isobutyramide			69–70
PM valeramide	53 09	52 05	74 5–75 5
PM isovaleramide	53 09	52 10–52 34	78–81
PM hexanamide			60–62
PM nonanamide	46 22	46 06	72 5–73 5
PM benzamide[a]			172–173
PM succinimide[a]			195–196 3
PM phthalimide[a]			212 5–213 5

[a] Compound not new

Polarography.—The Polarograph used was a Sargent Manual Model III The potentials were measured against the standard saturated calomel electrode At a pressure of 60 cm of mercury the drop time (t) was 3 2–3 6 sec /drop The Lingane and Laitinen (6) H cell was used Dissolved oxygen was removed by passing nitrogen through the test solution for 15 minutes The supporting electrolyte was 0 05 N $KClO_4$ Three drops of 0 01% gelatin were used to suppress the maxima

Preparation of Test Solutions—Stock solutions of the phenylmercury compounds were made up in water at a concentration of 12×10^{-4} M except in the case of the phthalimide compound where decreased water solubility made it difficult to work with levels above 4×10^{-4} M Fifty milliliters of the stock solution and 100 ml of 0 1 N $KClO_4$ were placed in a 200 ml volumetric flask, the pH adjusted to 7 0 with 0 1 N KOH and the solution diluted to the mark (final concentration 3×10^{-4} M) It was found that during the polarographic reduction there was a change of not more than 0 2 pH toward the acid side

Results and Discussion—All of the phenylmercury compounds studied were reductively decomposed in a two single electron step process similar to that demonstrated before by Benesch and Benesch (7) and Wuggatzer and Cross (8) for similar mercury compounds According to Benesch and Benesch (7) the two waves correspond to the two step reduction

$$RHgOH + H^+ + e \quad RHg^\bullet + H\ O$$
$$RHg^\bullet + H^+ + e \quad RH + Hg$$

In the first step which occurred at a voltage of 0 200–0 220 the hydroxide or phenylmercuric ion is reduced to the phenylmercury radical In the second step, the radical is decomposed into the hydrocarbon and free mercury ($E^1/_2 = -1\ 14$ v)

This reduction is reported to be irreversible (6) and one might expect it to be so since the phenyl mercury radical is formed It was reported by Benesch and Benesch (7) that the slopes of the waves varied with conditions such as concentration and pH and no simple relationship between potential and current (such as a linear plot of E *versus* log $i/(i_d - i)$) was found to describe the waves Also the phenylmercury radical and resulting reaction products are absorbed in the mercury surface and lead to some wave irregularities However, according to Lingane (9) the half wave potential is a characteristic of a compound even though the decomposition is not reversible and Calvin and Bailey (10) on this basis were able to make a comparison of the stability of a whole series of copper chelates

In this work a comparison was made of the half wave potentials of some phenylmercury amide and imide compounds It was found that all of the compounds were reductively decomposed in a two step process similar to that of the parent compound and the data reported here give the range of potentials obtained The work of Benesch and Benesch (7) and Wuggatzer and Cross (8) showed that there is considerable variation in the half-wave potentials of phenylmercury compounds with change in buffer, pH, and concentration of the mercurial For instance, Wuggatzer and Cross report values for the first half wave potential for phenylmercuric nitrate as −0 132 v at pH 7 0 in Britton-Robinson buffer and −0 247 v in Michaelis buffer system Under the condition of our experiments we obtained the results shown in Table II The value of the first half wave for phenylmercuric hydroxide varied from −0 20 to −0 22 v with an average of 0 210 v Among the phenylmercury amide and imide compounds there was somewhat more variation in the first half wave Values obtained varied from 0 200 v

for the phenylmercury benzamide and phenylmercury phthalimide to −0.245 v. for the isovaleramide and succinimide compounds. Since the averages for the half-wave potential for each compound were approximately the same as that of the phenylmercuric hydroxide and considering the overlapping in the range of values obtained for each compound it was concluded that not much significance could be attached to these variations. Accordingly, it is apparent then that these mercury amide and imide compounds dissociate readily in water to the hydroxide so that in each case the reductive decomposition of phenylmercuric hydroxide is measured.

This comparative study was repeated under a variety of conditions such as lower concentration of mercury compound, other carrying electrolytes as potassium acetate, and with buffer systems such as that prepared by Britton-Robinson. The same relative results were obtained but the potentials were shifted to lower values (−0.10 to −0.18 v. for the hydroxide) and the reproducibility of data was not as good. With potassium acetate as carrying electrolyte, high residual currents were obtained, probably because of the formation of mercuric acetate complexes. The results obtained in Britton-Robinson buffer agreed well with those of Benesch and Benesch (7) and Wuggatzer and Cross (8). A slight increase in the half-wave potentials was obtained for all the compounds with an increase in pH, suggesting the irreversibility of this reduction (7).

The second half wave of phenylmercury hydroxide was −1.145 v., an average of ten determinations. With the phenylmercury amide and imide compounds, there was considerable variation in this second half wave, values obtained under the condition of our experiments varying from −1.080 in the case of the propionamide compound to −1.225 v. for the valeramide compound or a range of 145 mv. Since this potential measures the same reaction in all cases, that is, the reductive decomposition of the phenylmercury radical, less variation might be expected. The overlapping of the ranges of values obtained suggests that the variations are not significant.

Phenylmercury hydroxide had an average wave height of 1.6 microamp. in the first wave and 3.61 microamp. in the second. This agrees with the average wave heights for the amide and imide compounds of 1.56 (range 1.48–1.93) and 3.83 (range 3.32–4.19 microamp.). The wave heights increased for all the compounds with increasing concentration of the mercurial but varied with the different buffers used and the acidity of the media.

In Table III is given data showing the effect of increasing concentration of the amides and imides on the half-wave potential of the first reductive decomposition of phenylmercuric hydroxide. It will be observed that with the amides of lower molecular weight there is no change in the half-wave potential even in molar ratios of amide to mercurial of 1,000:1. With the hexanamide there is a significant increase in negative potential (range −0.300 to −0.342 v.) at a molar ratio of amide to mercurial of 1,000:1 amounting to an average increase of 111 mv. A significant increase was also obtained with succinimide in a molar ratio of 100:1 (range of potential −0.255 to −0.262 v.) and for nonanamide in a molar ratio of 10:1. The effect of nonanamide

Table II.—First Half-Wave Potentials for Phenylmercury Amide and Imide Compounds

Compound	First Half-Wave Potential[a]
PMOH	0.200–0.220
PM acetamide	0.212–0.225
PM propionamide	0.212–0.240
PM valeramide	0.216–0.235
PM isovaleramide	0.226–2.245
PM hexanamide	0.200
PM nonanamide	0.220–0.225
PM benzamide	0.200–0.214
PM succinimide	0.220–0.245
PM phthalimide	0.200–0.220

[a] At least 10 determinations were made, in some cases 20

above this ratio could not be studied because of its insolubility in water.

Since the excess of amides and imides did not change the ionic strength of the media, nor apparently change the wave heights of the first or second wave and did not significantly change the potential of the second reductive decomposition it must be concluded that the effect of the hexanamide, nonanamide, and succinimide (and presumably phthalimide) was one of shifting the hydrolytic equilibrium toward the complex. How far the equilibrium is shifted is, of course, impossible to ascertain from these experiments. The lack of appearance of a third wave, one for the decomposition of the phenylmercuric amide or imide compound as was found by Benesch and Benesch (11) for glutathione and phenylmercury may indicate either that the position of this third wave is sufficiently close to that of the phenylmercuric hydroxide so that only a resultant wave was obtained under the condition of our experiments or that the shift of equilibrium by the excess of amides and imide was relatively small even with the large excess of complexing compound.

In either case it is evident that the amide compounds of lower molecular weight are readily hydrolyzed even with the amides present in large excess. This is also true for the amides of higher molecular weight and also the imides when present in solution with the phenylmercuric hydroxide in a molar ratio of 1:1. A rough parallelism seems to exist between the increase in negative half-wave potential and molecular weight of the complexing amide or imide, with the increase in organic solubility of the resulting phenylmercury amide and imide compound, and also with their decrease in water solubility.

DISCUSSION

K. L. Mandal studied the formation and stability of 3- and 4-coordinated compounds of mercury dibenzidine (12) and also 4-coordinated mercuric salts with diamines (13). He concluded from his work that mercury does not appear to form stable 4-coordinated compounds, hydrolyzing very readily in water to the more stable 3-coordinated compound. The one exception he found was the dibenzidine dipiperidine compound which was insoluble in water. Among the 3-coordinated compounds only the dibenzidine pyridine and piperidine compounds were stable in water. Diamines both aliphatic

TABLE III—EFFECT OF EXCESS CONCENTRATIONS OF AMIDES AND IMIDES ON THE HALF-WAVE POTENTIAL OF PHENYLMERCURIC HYDROXIDE

	Molar Ratios[a]			
	3×10^{-4} 1 1	3×10^{-3} 1 10	3×10^{-2} 1 100	3×10^{-1} 1 100
Acetamide	−0 219	−0 217	−0 217	−0 226
Propionamide	−0 216	−0 217	−0 214	−0 207
Valeramide	−0 220	−0 214	−0 203	−0 225
Hexanamide	−0 213	−0 218	−0 244	−0 326
Nonanamide	−0 232	−0 364 −(0 350–0 440)	.	−(0 300–0 342)
Succinimide	−0 222	−0 225	−0 258 −(0 255–0 262)	−0 270 −(0 250–0 280)
Phthalimide	−0 202		.	

[a] At least 9 determinations for each

and aromatic appear to form 4-coordinated compounds which are stable in organic solvents

Our work, in general, confirms that of Mandal. While it is comparatively easy to prepare the 2-coordinated compounds of phenylmercury such as the phenylmercuric amide and imide compounds, attempts to prepare amide and imide compounds in which the coordination was 3 and 4 were unsuccessful. Reactions were attempted by heating in several organic solvents ($CHCl_3$, acetone, alcohol, ether) and also by melting the compounds together. The 2-coordinated compound was obtained on crystallization.

Preparation of phenylmercury compounds of bi and tridentate chelating agents such as glycine, β-alanine, γ-aminobutyric acid, ethylene diamine, diethylene triamine, glycylglycine, and EDTA (see table IV) were successful but no evidence could be obtained polarographically that chelation took place EDTA added to a phenylmercuric hydroxide solution in molar ratios of 1 1 and 10 1 had a $E^1/_2$ of 0 240 or about 0 02 v. more electronegative than phenylmercuric hydroxide, thus indicating only a slightly greater stability in water solution. However, in a 50 50 (v/v) pyridine water solution with 0 1 M potassium nitrate as carrying electrolyte, phenylmercuric γ-aminobutyric acid had a $E^1/_2$ about 375 mv. more negative than phenylmercuric hydroxide.

TABLE IV—MELTING POINT OF SOME POSSIBLE PHENYLMERCURY CHELATE COMPOUNDS

Compound	Melting Point, °C	Possible Coordination of Resulting Compound
PM β-alanine	195–215d	3
PM γ-aminobutyric acid	166–170d	3
PM glycine	120–124	3
PM glycylglycine	162–163d	4
PM diethylene triamine	122–123	4
PM EDTA		4

While phenylmercury may form stable chelates in organic solvents it is evident that among the compounds studied the complexes of phenylmercury are readily hydrolyzed as shown from this study The ready dissociation of all the amide and imide compounds observed in this study partially confirm the conclusions of Smalt, *et al.* (1), who found that phenylmercuric acetamide and benzamide were equally as effective as phenylmercuric hydroxide as biological inhibitors and who also found a ready dissociation of these compounds in chromatographic studies with neutral solvents. The results of this study are not consonant with their conclusion as to the lesser dissociation of the phenylmercuric succinimide and phthalimide compounds.

From these polarographic studies it is evident that the decreased biological activity of the phthalimide and succinimide cannot be explained on the basis of greater stability. Possibly a greater lipoid solubility or a decreased water solubility may play a part in determining the germicidal efficacy. It was observed in this study that the formation of the amide and imide complexes greatly decreased the solubility of the phenylmercury in water and increased its solubility in organic solvents and it was noticed also that this solubility effect increased with increasing molecular weight of the complexing ligand.

From the data presented it seems that the stability of the phenylmercuric imides is no greater than that of the amides unless one compares the imides with the amides of lower molecular weight This observation is only partially in agreement with the findings of Whitmore (14) from measurements of conductivity that the Hg—N linkage of imides is more stable than the Hg—N of amides.

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Studies on Antibacterial Vapors of Volatile Substances*

By THOMAS C. GRUBB

Two simple methods are described for making a quantitative estimate of the bacteriostatic and bactericidal action of vapors from volatile substances. One method is used for static exposure periods of twelve to eighteen hours, the other is for dynamic exposure periods as short as one second. The results of screening twenty-two compounds by the former method are described. The types of bacteria commonly associated with respiratory infections were employed as test organisms. It is interesting to observe that many of the drugs which have been employed empirically many years for the inhalation therapy of respiratory infections display a measurable degree of *in vitro* antibacterial and antiviral activity.

FOR CENTURIES mankind has sought to alleviate and cure its sufferings from many types of diseases by the inhalation of vapors from volatile oils, incense, etc. It seemed reasonable to believe that if the disease was localized in the respiratory tract or if the most severe symptoms were confined to this area, the most direct attack on the source of the disease was by the inhalation of some volatile medication. As empiricism gave way to the scientific method, bacteriologists began to test the volatile substances which folklore had credited with therapeutic value. Thus within a comparatively short time, a number of publications appeared which clearly demonstrated by accepted techniques that the vapors from a number of substances were able to inhibit the growth or actually kill many different types of pathogenic bacteria and some viruses. In 1920 Macht and Kunkel (1) showed that the vapors from myrrh, cinnamon, incense etc. exhibited distinct bacteriostatic but slight bactericidal action on *Escherichia coli* and *Pseudomonas pyocyaneus*. Morel and Rochaix (2) exposed meningococci, typhoid bacilli, staphylococci, diphtheria bacilli and anthrax spores to the vapors of various essential oils. Of these, the oils of citron, thyme, and orange were found to be germicidal after fifteen minutes contact for some species while other species required as long as eight hours exposure. However, they were unable to kill anthrax spores under any of their experimental conditions. Schobl and Kusana (3) showed that vapors from a number of essential oils had a bacteriostatic effect on the tubercle bacillus, *Vibrio cholerae*, and staphylococci. Walker, *et al* (4), noted that vapors from onions possessed bactericidal activity. In 1927 Wrenn (5) irradiated cod liver oil, cottonseed oil, olive oil etc. and concluded that secondary germicidal emanations were produced from these oils. However, five years later, Harris, *et al* (6), demonstrated that Wrenn had incorrectly interpreted his findings since the germicidal action of the irradiated oils resulted from the production of volatile peroxides. These same workers (7) also studied the effect of irradiating various fish and vegetable oils in the production of antiseptic vapors. After a fifteen minute irradiation period, cod liver oil vapors inhibited *Streptococcus epidemicus*, *Eberthella typhosa*, *E. coli*, etc. Moreover, if these oils were irradiated for twenty-five minutes, then their vapors prevented the germination of *Clostridium sporogenes* and *Bacillus subtilis* spores. The antibacterial activity of garlic vapors was studied by Walton and his co-workers (8) who reported that expsoure for periods of four to one hundred twenty minutes at 37° was inhibitory for *Mycobacterium butyricum*, *Mycobacterium smegmatis*, and *Serratia marcescens*. In another publication by McKnight and Lindegren (9), *Mycobacterium leprae* was inhibited after one hour's exposure to garlic vapors at 37°. The germicidal activity of these vapors was attributed to their acrolein content by Vollroth, *et al* (10).

In 1940 Lebduska and Pidra (11) published the most extensive study on the antibacterial action of vapors reported in the literature up to that point. These workers streaked plates with *E. coli* and *S. aureus* and exposed them to the vapors of a variety of substances, many of which displayed bacteriostatic or bactericidal action. In the same year Foter (12) tested the antibacterial activity of vapors from allyl isothiocyanate and related oils. He concluded that the bacteriostatic action of garlic vapors was due to their allyl isothiocyanate content.

The vapors from cinnamon and eucalyptus oils were found by Remlinger and Bailly (13) to be bactericidal for the typhoid and paratyphoid bacilli as well as neutralizing the viruses of rabies, pseudorabies, and equine encephalo-

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myelitis. In China, Chu and Pai (14) described the bacteriostatic action of garlic vapors for *Staphylococcus aureus* after ten minutes contact and *E. typhosa* after twenty minutes contact at 37°. In Russia, Tokin (15) studied the antibacterial action of vapors from various plants and concluded that the vapors from onion and garlic were the most potent. Tokin found that an onion paste ceased to produce bactericidal vapors ten to fifteen minutes after it was prepared. The mechanism of the antibacterial vapors from cod liver oil was further clarified by Ross and Poth (16) who demonstrated that fresh oil did not produce antiseptic vapors against *S. aureus*, *B. subtilis*, and *E. coli*, but that oil which had been exposed to the air or had its natural antioxidants removed did give off antiseptic vapors. Their results suggested that the germicidal principle in the vapors was acrolein.

EXPERIMENTAL

The purpose of this report is to describe *in vitro* methods for making a quantitative evaluation of the antibacterial activity of the vapors of volatile compounds. Attention was directed primarily to compounds which might be used therapeutically rather than to substances which are used on inanimate objects, e. g., ethylene oxide, beta propiolactone, triethylene glycol, etc.

When it is not important to determine how rapidly vapors inhibit or kill bacteria, a static technique may be used as follows. Exactly 20 cc. of nutrient agar are poured into Petri dishes, and after the medium has solidified, one drop of a 1:100,000 dilution of *S. aureus* is placed on the agar and spread uniformly over the surface with a sterile glass rod. The inoculated medium is then inverted over the top of the cover of the Petri dish which contains the substance to be tested. There are a number of ways that the vapors from the test substance may be evaluated, however, it is important to remember that where quantitative tests are being carried out for comparative or control purposes, it is essential that the quantity and area of the exposed surface of the test substances be identical in all tests. Best results have been obtained by annealing glass rings 20 mm. in diameter and 0.5 mm. high in the exact center of the inner surface of a Petri dish top. The substance to be tested is then placed in this cup and the inoculated bottom inverted over it. Less satisfactory is the application of the test substance to a filter paper circle approximately 4 mm. in diameter which is placed in the center of the dish cover. If the test substance is a powder, it may be dissolved in some nonvolatile solvent, the filter paper then dipped into it and placed on the inner surface of the Petri dish top. Volatile solvents may be used provided a control with the solvent alone is tested, and if it also produces antibacterial vapors, the effect of these vapors must be taken into account in determining the net effect of the compounds under test.

Since a considerable variation in plate counts is to be expected, it is essential to employ a sufficient number of replicate plates so that the results will be statistically significant. After the plates have been prepared, they are placed in the 37° incubator for eighteen to twenty-four hours before counting. The covers are then removed and replaced with fresh sterile tops not containing the test substance. These plates are then returned to the incubator for an additional eighteen to twenty-four hours. The first count may be considered the "bacteriostatic" count and the second, the "bactericidal" count, since colonies often appear after forty-eight hours which were repressed during the initial incubation period. One additional test may be carried out to be certain that the absence of growth after the forty-eight hours incubation period is the result of bactericidal action and not merely bacteriostasis caused by absorption of the vapors into the medium. A sterile cork borer 1.0 cm. in diameter is used to remove agar cores from the plates where no growth has developed (while the control plates show considerable growth in a corresponding area). Each core is transferred aseptically to a flask containing 250 cc. of nutrient broth and incubated at 37° for one week. If no visible growth develops, it is reasonable to assume that no viable bacteria were present in the agar sample tested. Using the above methods, many variations may be introduced according to the needs of the problem. For example, different organisms, media, and concentrations of the inoculum may be employed. Table I shows not only the bacteriostatic and bactericidal action of 0.5% chlorothymol mixed with other essential oils, but also indicates a vast difference in the resistance of two different strains of *S. aureus* to these vapors.

TABLE I.—BACTERIOSTATIC AND BACTERICIDAL ACTION OF CHLOROTHYMOL VAPORS ON *S. aureus*

Test Substance	Total Number of Colonies per Plate: 24 Hr. Incubation, M Strain	24 Hr. Incubation, B Strain	48 Hr. Incubation, M Strain	48 Hr. Incubation, B Strain
Control	265	330	265	354
	222	352	225	360
	252	293	260	300
	93	308	93	310
	225		225	
Mean	211	321	213	331
0.5% Chlorothymol	259	0	270	4
	246	0	246	13
	212	0	220	4
	232	0	235	0
	280	0	280	8
Mean	246	0[a]	250	6[a]

[a] Agar plugs cultured from areas without colonies did not show any growth after seven days incubation at 37°.

In Fig. 1 the number of colonies and their distribution when a resistant and susceptible strain of *S. aureus* are exposed to the vapors of 0.5% chlorothymol for twenty-four hours may be observed.

Using the static technique, over 50 different substances were screened for their antiseptic vapor action, with *S. aureus* and *Klebsiella pneumoniae* as the test organisms. In Table II only those substances which showed some activity against either of the test organisms are recorded.

These results indicate that many more substances are active against *S. aureus* than against *Kl. pneumoniae*, and if one substance is effective against both

TABLE II —COMPARATIVE ANTIBACTERIAL VAPOR ACTION OF VARIOUS SUBSTANCES ON *S aureus* and *Kl pneumoniae*

Inhibition of Growth, %	Test Organism	Substance[a]
75–100	*S. aureus*	Salicylaldehyde
		Oxyquinoline
		Chlorothymol
		Oil of mustard
	Kl pneumoniae	Oxyquinoline
25–75	*S aureus*	Thymol
		Lauryl alcohol
		Phenyl isothiocyanate
	Kl pneumoniae	None
25–50	*S. aureus*	Oxyquinoline benzoate
		Methyl cinnamate
		Caproic acid
		Turpentine
	Kl. pneumoniae	Oxyquinoline benzoate
		Salicylaldehyde
5–25	*S. aureus*	Oil of cloves
		Oxalidine
		Menthol
		Chlorobutanol
		Beta pinene
		Decyl alcohol
		Camphene
		Oleoresin of capsicum
	Kl pneumoniae	Chlorothymol

[a] Dilutions of 1–5% in triethylene glycol or petrolatum

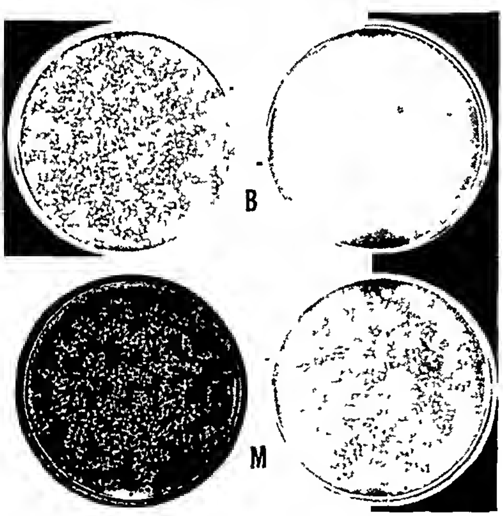

Fig 1 —Comparative resistance of *S aureus* strains Number of colonies developing in control (left) and 0 5% chlorothymol (right) static vapor test after twenty-four hours exposure Note difference between susceptible B strain (upper) and resistant M strain (lower).

organisms, it is usually more effective against *S aureus*

In addition to the testing of antibacterial vapors under static conditions, it may be important to test them under dynamic conditions in which the vapors pass rapidly across the surface of the test organisms for various time intervals The apparatus employed for this purpose is shown in Fig. 2.

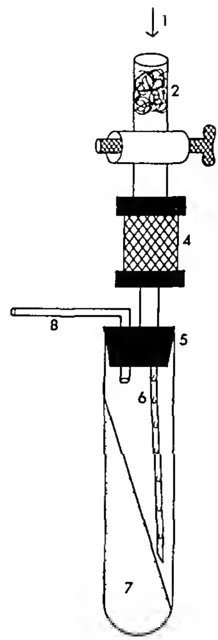

Fig 2.—Diagram of apparatus employed in the dynamic vapor exposure tests. 1, air inlet; 2, cotton air filter; 3, stopcock; 4, gauze impregnated with test substance; 5, two-hole rubber stopper; 6, copper tube with pinholes, 7, agar slant; and 8, air outlet

Through one hole of a two-hole rubber stopper is passed a copper tube (0 2 mm diam.) 10 cm. long closed at the distal end and having "pin" holes (0 1 mm diam) every 2 cm along the length of the tube; and through the other hole is passed a glass tube 5 mm in diameter The stopper is inserted tightly into the end of a test tube containing an inoculated agar slant Sterile air is drawn through the tube containing cotton gauze impregnated with the substance to be tested and into the copper tube inserted into the culture tube in such a way that the pin holes are facing the agar slant, and the air flows uniformally across the inoculated surface of the agar The outlet tube is connected to a flowmeter. The air flow is adjusted to 17 5 cc /second and by means of the stopcock, the inoculated medium can be exposed to vapors for any time interval from one second upward

After the inoculated tube has been treated with the flowing vapors for the desired period, it is removed from the two-hole stopper and the sterile cotton plug replaced with aseptic precautions After all the tubes in a given experiment have been exposed to the vapors, they are incubated at 37° for twenty-four hours and the number of developing colonies counted. Any reduction in the number of colonies compared to the air control indicates a bacteriostatic or bactericidal action. The results of a typical experiment with the very effective oxyquinoline vapors and ineffective triethylene glycol after thirty to one hundred and twenty seconds contact are shown in Fig 3

The results of testing the dynamic vapors effect on other types of bacteria associated with respiratory infections are shown in Table III.

TABLE III.—ANTIBACTERIAL ACTION OF OXYQUINOLINE VAPORS ON VARIOUS RESPIRATORY BACTERIA

Test Organism	Exposure	Total number of colonies per tube 30 sec.	60 sec.	90 sec.	120 sec
S. aureus	Air control	338	356	356	492
	Oxyquinoline	117	50	0	0
Pneumococcus	Air control	48	52	60	40
Type III	Oxyquinoline	28	1	1	0
Hemolytic	Air control	...[a]	106	126	166
streptococcus	Oxyquinoline	...	26	0	0
H. influenzae	Air control	87	73	97	63
	Oxyquinoline	64	34	50	21

[a] Test not completed.

Fig. 3.—Dynamic vapor tests on *S. aureus*. Number of colonies developing in air control (left), triethylene glycol (center), and oxyquinoline (right), after thirty to one hundred twenty seconds exposure to vapors and subsequent incubation for twenty-four hours at 37°. Time of exposure in seconds is shown below each tube.

The results illustrate how rapidly certain vapors inhibit or kill bacteria under the conditions of this test. Of course, to be certain that the absence of growth results from bactericidal rather than bacteriostatic action, subculture tests, similar to those described above, must be carried out.

It is evident that by employing the above described static and dynamic vapor tests for detecting the antibacterial action of volatile substances, it is possible to make a valid quantitative comparison of their bacteriostatic and/or bactericidal action. By means of these tests, many substances have been screened for their potential therapeutic action.

SUMMARY

By means of suitable quantitative experiments it was found possible to screen vapors from volatile compounds for their action on bacteria. It is thought-provoking to observe that a number of vapor-producing compounds which have been used for many years for the inhalation therapy of minor respiratory diseases (thymol, menthol, turpentine, oil of mustard, chlorothymol, etc.) have been shown to liberate vapors which are bactericidal for the types of organisms most frequently associated with respiratory infections, and also display a measurable degree of *in vitro* antiviral action against the influenza virus (17).

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The Identification and Determination of Some Official Local Anesthetics as Tetraphenylborates*

By L. G. CHATTEN, M. PERNAROWSKI, and L. LEVI

The tetraphenylborates of some official local anesthetics may be prepared by interacting the hydrochlorides of the organic bases with sodium tetraphenylboron in aqueous citrate buffers at a pH of 6.0. The reaction affords a high degree of sensitivity, permitting the detection and quantitative estimation of these drugs. The ultraviolet spectra of the derivatives depend, mainly, on the spectral characteristics of the organic bases. Infrared measurements are presented to confirm the mechanism of reaction and to illustrate the value of the IR data for identification purposes.

INTO THAT class of drugs termed local anesthetics are incorporated three general groups of organic compounds: phenols, alcohols, and certain benzoic acid esters of short chain alcohols. Although important, the first two broad groups are overshadowed by the importance of the third group, which includes dibucaine, piperocaine, procaine, and tetracaine hydrochlorides as official drugs in the United States Pharmacopeia as well as naepaine hydrochloride and butethamine hydrochloride which are official in the National Formulary.

Although the official compendia prescribe certain tests and standards for these local anesthetics, new methods for their identification and analyses in pharmaceutical and toxicological materials are continuously being sought (1–5). Several years ago, Wittig and his co-workers found that the tetraphenylboron ion $[B(C_6H_5)_4]^-$ formed compounds with potassium, ammonium, rubidium, and cesium (6, 7, 8), which are very insoluble in water. This observation and subsequent reports that the ion also reacted with basic organic nitrogen compounds led to widespread investigations into the usefulness of the reagent in qualitative and quantitative analyses. Comprehensive literature reviews on the subject have been published by Gloss (9) and Barnard (10, 11, 12).

While the reactions of a wide variety of basic organic nitrogen compounds with sodium tetraphenylboron have been reported in the literature, the local anesthetics have not been extensively investigated. Gautier, Renault, and Pellerin (13) prepared the tetraphenylborates of procaine, tetracaine, and dibucaine. In addition, Schultz and Mayer (14) reported that this reagent would react with certain local anesthetics. Fischer and Karawia (15) reported the preparation of procaine tetraphenylborate and stated the melting range to be 147–151°. Flaschka, Holasek, and Amin (16) showed that the reaction of the tetraphenylboron ion with mercuric chloride resulted in the release of hydrochloric acid. This reaction was used to determine either potassium or various basic organic nitrogen derivatives of tetraphenylboron. Procaine was included in the compounds investigated.

METHOD

Apparatus.—Beckman pH meter, model G; Beckman glass electrode no. 1190–80; Beckman calomel electrode no. 1170–71; Fisher-Johns melting point apparatus; Cary recording spectrophotometer, model 10; Beckman DU spectrophotometer; Perkin-Elmer infrared spectrophotometer, model 21; and Micro-Kjeldahl apparatus (Scientific Glass Co.).

Reagents and Solutions.—Dibucaine HCl, U.S.P.; piperocaine HCl, U. S. P.; procaine HCl, U. S. P.; tetracaine HCl, U. S. P.; butethamine HCl, N. F.; naepaine HCl, N. F.; methanol AR, obtained from Mallinckrodt Chemical Works; petroleum ether, A. C. S.; acetone, A. C. S.; glacial acetic acid, A. C. S.; sodium tetraphenylboron solution 2% as prepared by Cooper (17); citrate buffers of pH 3, 4, 5, and 6 as described by Clark (18); crystal violet indicator solution prepared by dissolving 500 mg. in 100 ml. of glacial acetic acid; acetous perchloric acid 0.1 *N*; and acetous perchloric acid 0.05 *N*.

PROCEDURES

Establishment of Purity of Local Anesthetics.—Each compound was recrystallized by dissolving 10 Gm. in 25 ml. of ethanol with the aid of heat. The solution was filtered while hot and cooled for thirty minutes in a refrigerator. Crystals were collected on a sintered-glass funnel and dried at 100°. Melting points were determined and nonaqueous titrations carried out potentiometrically in anhydrous methanol using a glass calomel electrode system. The titrant was 0.1 *N* acetous perchloric acid.

Preparation of the Tetraphenylborates of Local Anesthetics.—Add to an accurately weighed sample of about 0.2 Gm. of the local anesthetic or to an accurately measured aliquot of the pharmaceutical product to be analyzed, 20 ml. of citrate buffer (pH 6) and, dropwise, 2% sodium tetraphenylboron solution. Stir the solution well during the addition of the reagent and add approximately 1 ml. excess. Set the mixture aside for about one hour and collect

* Received April 25, 1958, from the Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada

The authors express their appreciation to Mr. W. Skakum for his assistance with the ultraviolet and infrared spectra, and to Mr. G. Morris for his preparation of the figures

the precipitate under suction in a sintered-glass crucible of fine porosity. Dry in a vacuum desiccator over phosphorus pentoxide for twenty-four hours.

Volumetric Analysis of Tetraphenylborates.—Dissolve approximately 100 mg. of the local anesthetic tetraphenylborate in 5 ml. of acetone. Add 45 ml. of glacial acid and one drop of crystal violet indicator. Titrate to a blue end point for piperocaine and dibucaine tetraphenylborates and to a green end point for the tetraphenylborates of procaine, naepaine, and tetracaine.

Ultraviolet Measurements.—The spectra of both the hydrochloride salts and the tetraphenylborates were taken in anhydrous methanol. The final dilution was such that concentration, in all instances, ranged from 1 to 2 mg. in 100 ml. of solvent. No heat was used during the preparation of the solutions. Absorbances were determined in 1-cm. cells using either the Beckman DU or Cary recording spectrophotometer. From the data so obtained, the log ϵ values were calculated and plotted against wavelength.

Preparation of Samples for Infrared Examination.—The infrared spectra of the local anesthetics and their tetraphenylborates shown in Fig. 3 were recorded on a Perkin-Elmer Double-Beam Model 21 Spectrophotometer equipped with sodium chloride optics.

The materials were finely powdered in a mechanical grinder until they would pass through a 250-mesh sieve (U. S. Standard Sieve Series No. 230). Accurately weighed amounts were then mixed intimately with sufficient reagent grade potassium bromide A. C. S., treated similarly and dried overnight at 125° to obtain preparations of 0.5% w/w composition. Aliquots of 200 mg. were subjected *in vacuo* to a pressure of 10,000 pounds/sq. inch for about five minutes and the absorbances of the clear disks thus produced measured over the frequency range extending from 4,000–650 cm.$^{-1}$. A potassium bromide disk prepared under comparable conditions was placed in the path of the reference beam to compensate for absorption by the reagent. After each experiment the disks were weighed in order to determine the amount of sample present. The weight variations between different disks never exceeded 1%.

DISCUSSION

The melting points of the purified local anesthetics and the results of the nonaqueous titrations of these compounds are reported in Table I. It is seen from this data that the compounds meet the requirements of purity as established by the "United States Pharmacopeia" (19) and the "National Formulary" (20)

Table I.—Criterion of Purity of Local Anesthetics

Compound	Melting Range, °C.	Purity by Nonaqueous Titration, %
Butethamine HCl	193.9–194.6	99.9
Dibucaine HCl	98.8–100.0	100.1
Naepaine HCl	177–178	99.9
Piperocaine HCl	175	100.4
Procaine HCl	156.5–157.6	99.4
Tetracaine HCl	147.5–149.9	98.6

The tetraphenylborates were prepared at pH 3, 4, 5, and 6, in accordance with the procedure described. Because of reports that increased temperatures, under certain conditions, decompose these derivatives (8, 21), the solutions were not heated. It was found that recoveries were essentially the same within a pH range of 3 to 6, provided the sodium tetraphenylboron solution was added slowly and with stirring to prevent co-precipitation of the citrate ions from the buffer. On recovery of the precipitates, there appeared to be little difference in their physical appearance. In general, however, finer precipitates were obtained when the pH of the initial solution was either 5 or 6. On standing, the precipitates, prepared at pH 3 or 4, darkened slightly and developed phenolic-like odors. Only piperocaine tetraphenylborate appeared to be unaffected by pH throughout the range investigated. Percentage recoveries of the tetraphenylborates, prepared at pH 6, are recorded in Table II. Several commercial intravenous pharmaceuticals were also assayed by the proposed procedure and comparative analyses carried out using the official method of the "United States Pharmacopeia" (19). From the data reported in Table III, it is noted that satisfactory agreement was obtained between the two methods.

Using the procedures described in this paper, butacaine tetraphenylborate could not be recovered quantitatively. Excessive clumping resulted in the formation of hard amber lumps and recoveries were erratic. The reaction was studied also with xylocaine, cyclomethycaine, tronethane, larocaine, hexylcaine, panthesin, and diothane. Generally, recoveries of the tetraphenylborates were low, in these instances. No correlation between the chemical structure of the compounds investigated and their recoveries could be detected. Evidently other factors also influence the course of the reaction.

Crane (22) reported that the original tetraphenylborates were quite pure and required no recrystallization. Gautier, Renault, and Pellerin (13) established the purity of the individual derivatives by titrating them in glacial acetic acid with acetous perchloric acid. The indicator was crystal violet. Their technique, with slight modifications, was employed in this laboratory to determine the purity of the tetraphenylborates. From the results of these titrations, as given in table IV, it would appear that the compounds are of such high purity as to obviate the necessity of recrystallization.

Ultraviolet Data.—Results of the ultraviolet studies are shown in Figs. 1 and 2 and summarized in Table V. It was found that, unlike the reagent, the local anesthetics and their tetraphenylborates exhibit marked absorption in the ultraviolet region. It is concluded, therefore, that the absorption of the tetraphenylborate is due primarily to the contribution afforded by the local anesthetic. Thus ϵ maximum values for the hydrochlorides and their tetraphenylborates are in close agreement in every instance. For dibucaine and piperocaine the λ maximum of the hydrochloride and the tetraphenylborate occur at identical wavelengths. However, for procaine, naepaine, tetracaine, and butethamine the λ maximum of the tetraphenylborates are shifted by about 4–5 mμ toward shorter wavelengths.

The ϵ minimum values of the tetraphenylborates are, in every instance, considerably higher than those

The Identification and Determination of Some Official Local Anesthetics as Tetraphenylborates*

By L. G. CHATTEN, M. PERNAROWSKI, and L. LEVI

The tetraphenylborates of some official local anesthetics may be prepared by interacting the hydrochlorides of the organic bases with sodium tetraphenylboron in aqueous citrate buffers at a pH of 6.0. The reaction affords a high degree of sensitivity, permitting the detection and quantitative estimation of these drugs. The ultraviolet spectra of the derivatives depend, mainly, on the spectral characteristics of the organic bases. Infrared measurements are presented to confirm the mechanism of reaction and to illustrate the value of the IR data for identification purposes.

Into that class of drugs termed local anesthetics are incorporated three general groups of organic compounds: phenols, alcohols, and certain benzoic acid esters of short chain alcohols. Although important, the first two broad groups are overshadowed by the importance of the third group, which includes dibucaine, piperocaine, procaine, and tetracaine hydrochlorides as official drugs in the United States Pharmacopeia as well as naepaine hydrochloride and butethamine hydrochloride which are official in the National Formulary.

Although the official compendia prescribe certain tests and standards for these local anesthetics, new methods for their identification and analyses in pharmaceutical and toxicological materials are continuously being sought (1–5). Several years ago, Wittig and his co-workers found that the tetraphenylboron ion $[B(C_6H_5)_4]^-$ formed compounds with potassium, ammonium, rubidium, and cesium (6, 7, 8), which are very insoluble in water. This observation and subsequent reports that the ion also reacted with basic organic nitrogen compounds led to widespread investigations into the usefulness of the reagent in qualitative and quantitative analyses. Comprehensive literature reviews on the subject have been published by Gloss (9) and Barnard (10, 11, 12).

While the reactions of a wide variety of basic organic nitrogen compounds with sodium tetraphenylboron have been reported in the literature, the local anesthetics have not been extensively investigated. Gautier, Renault, and Pellerin (13) prepared the tetraphenylborates of procaine, tetracaine, and dibucaine. In addition, Schultz and Mayer (14) reported that this reagent would react with certain local anesthetics. Fischer and Karawia (15) reported the preparation of procaine tetraphenylborate and stated the melting range to be 147–151°. Flaschka, Holasek, and Amin (16) showed that the reaction of the tetraphenylboron ion with mercuric chloride resulted in the release of hydrochloric acid. This reaction was used to determine either potassium or various basic organic nitrogen derivatives of tetraphenylboron. Procaine was included in the compounds investigated.

METHOD

Apparatus.—Beckman pH meter, model G; Beckman glass electrode no. 1190–80; Beckman calomel electrode no. 1170–71; Fisher-Johns melting point apparatus; Cary recording spectrophotometer, model 10; Beckman DU spectrophotometer; Perkin-Elmer infrared spectrophotometer, model 21; and Micro-Kjeldahl apparatus (Scientific Glass Co.).

Reagents and Solutions.—Dibucaine HCl, U.S.P.; piperocaine HCl, U. S. P.; procaine HCl, U. S. P.; tetracaine HCl, U. S. P.; butethamine HCl, N. F.; naepaine HCl, N. F.; methanol AR, obtained from Mallinckrodt Chemical Works; petroleum ether, A. C. S.; acetone, A. C. S.; glacial acetic acid, A. C. S.; sodium tetraphenylboron solution 2% as prepared by Cooper (17); citrate buffers of pH 3, 4, 5, and 6 as described by Clark (18); crystal violet indicator solution prepared by dissolving 500 mg. in 100 ml. of glacial acetic acid; acetous perchloric acid 0.1 *N*; and acetous perchloric acid 0.05 *N*.

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TABLE II —MELTING RANGES AND PER CENT RECOVERIES OF TETRAPHENYLBORATES OF SOME OFFICIAL LOCAL ANESTHETICS AT pH 6

Procaine Melting Range °C	Procaine Recovery, %	Piperocaine Melting Range, °C	Piperocaine Recovery, %	Naepaine Melting Range, °C	Naepaine Recovery, %	Tetracaine Melting Range, °C	Tetracaine Recovery, %	Dibucaine Melting Range, °C	Dibucaine Recovery, %	Butethamine Melting Range, °C	Butethamine Recovery, %
141–143	99 8	113–116	100 3	110 5–112 5	99 5	119–120	99 2	115 5–118	99 8	91 5–108	Not reproducible
	99 9		99 1		100 7		100 4		100 6		
	99 3		99 2		99 3		99 6		99 1		

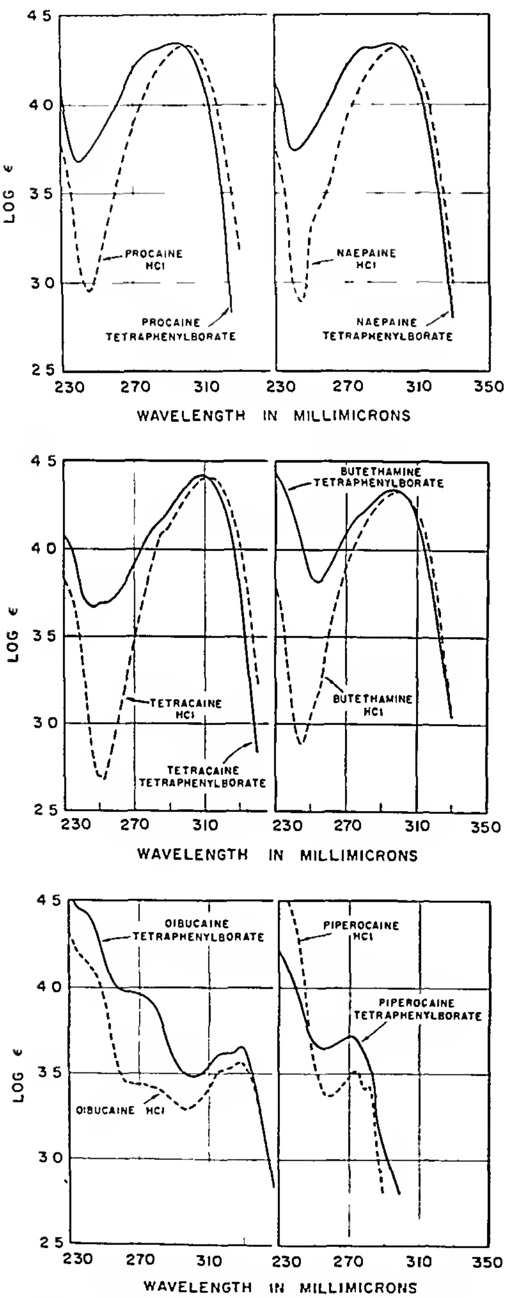

Fig 1 —Ultraviolet absorption spectra of some local anesthetics and their tetraphenylborates

TABLE III —RECOVERY OF PROCAINE FROM INTRAVENOUS PHARMACEUTICALS BY U S. P. METHOD OF ANALYSIS AND AS TETRAPHENYLBORATE

Manufacturer	Product	Recovery, % Tetraphenylborate	Recovery, % U S P
A	Procaine HCl 2%	103 0	100 4
		102 3	101 9
A	Procaine HCl 2% with epinephrine	99 5	98 3
		99 4	98 6
B	Procaine HCl 2%	101 7	100 4
		101 5	98 3
B	Procaine HCl 2%	98 8	97 6
		98 4	
C	Procaine HCl 2%	96 0	95 8
		95 1	95 2
C	Procaine HCl 2%	95 4	94 9
		94 2	94 6
D	Procaine HCl 2%	99 6	99 3
		99 2	99 3

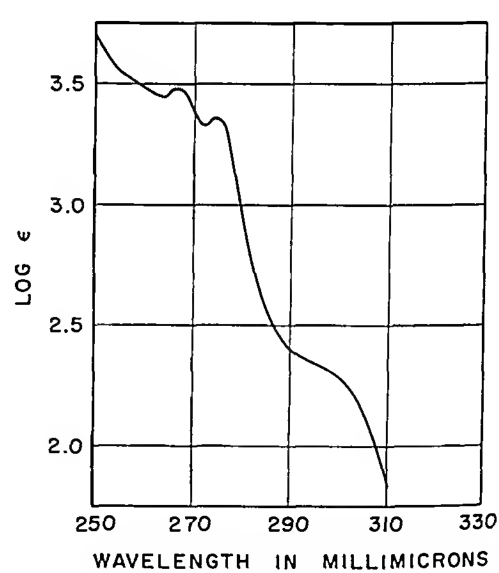

Fig 2 —Ultraviolet absorption spectrum of sodium tetraphenylboron.

of the corresponding hydrochlorides. The phenomenon is due, obviously, to the effect exerted by the tetraphenylboron moiety in the molecule In no instance does the λ minimum of the tetraphenylborate coincide with that of the hydrochloride In some instances, the former occurs at a slightly higher wavelength and in others at a slightly lower one

As one would anticipate from the similarity of

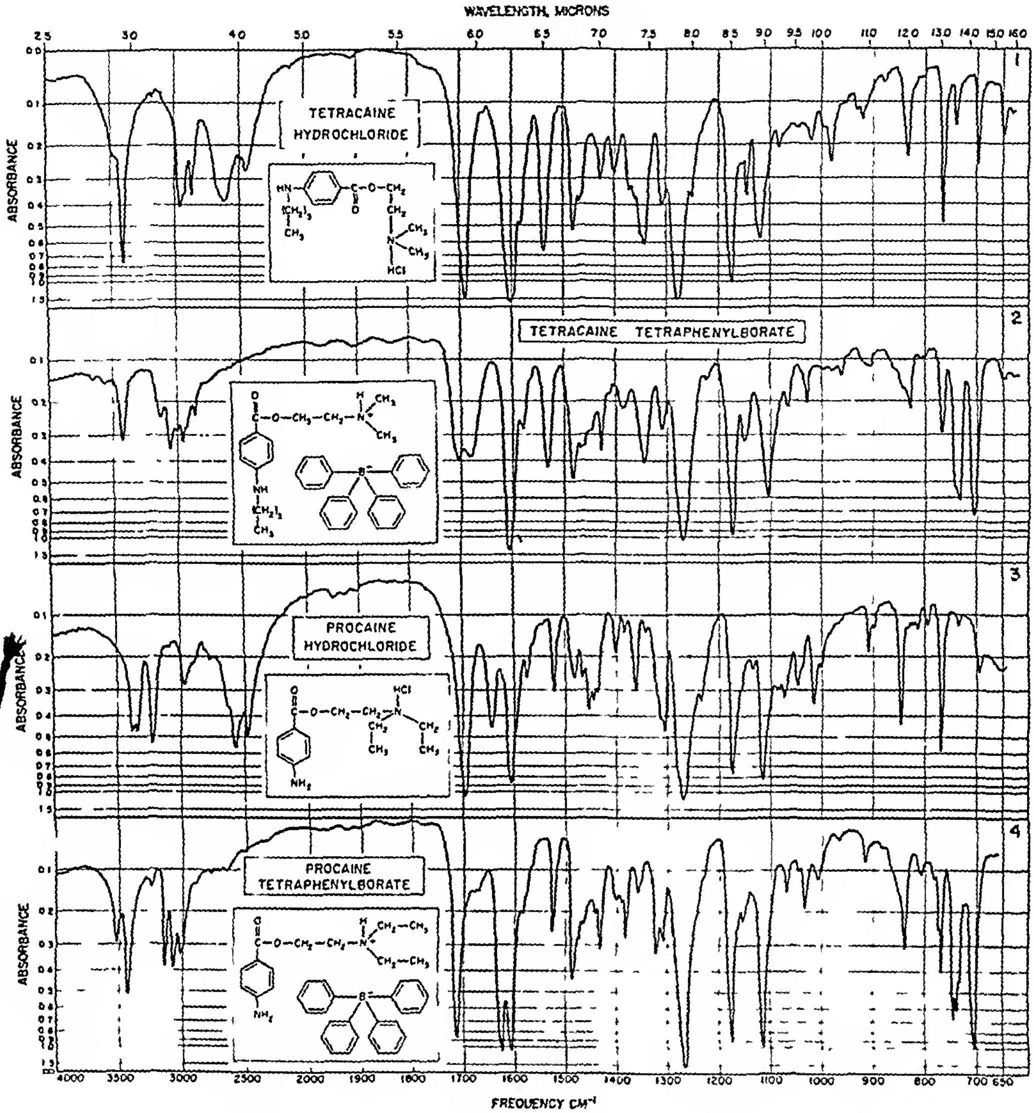

Fig. 3.—Infrared spectra of some local anesthetics and their tetraphenylborates.

their structural formulas, the spectra of procaine, naepaine, and tetracaine are remarkably alike. The λ maximum of the first two compounds occur so closely together, at 299 to 300 mμ, that it is impossible to differentiate between them. Tetracaine can, on the other hand, be distinguished by the fact that its maximum occurs at 314 mμ. The λ minimum of butethamine tetraphenylborate is of value in distinguishing it from procaine and naepaine. For butethamine, it occurs at 253 mμ whereas the λ minimum of the latter two compounds is observed at 240 and 241 mμ, respectively. With this exception, the tetraphenylborate spectra appear to have no advantage over those of the hydrochlorides. It appears that none of the spectra will permit differentiation of procaine and naepaine.

Both dibucaine and piperocaine can be differentiated readily from each other and from the remainder of the local anesthetics considered in this report by either their hydrochloride or tetraphenylborate spectra. This is to be expected however, when one considers the differences in their chemical structure. Dibucaine contains a quinoline group and piperocaine the piperidine moiety.

Infrared Data.—The infrared spectra of the compounds are shown in Fig. 3. The spectrum of sodium tetraphenylborate (Fig. 4) displays two intense bands in the 700–750 cm.$^{-1}$ region (out-of plane C-H bending vibrations of the phenyl groups) and in the C = C stretching region two marked absorptions are observed in the 1,500 and 1,600 cm.$^{-1}$ wavelength range, respectively (C = C skeletal inplane vibrations). Characteristic = C-H stretching vibrations occur in the form of a doublet throughout the 3,000-cm.$^{-1}$ region.

Comparison of the spectra of the local anesthetics with those of the tetraphenylborates shows that the reaction proceeds in accordance with the equati-

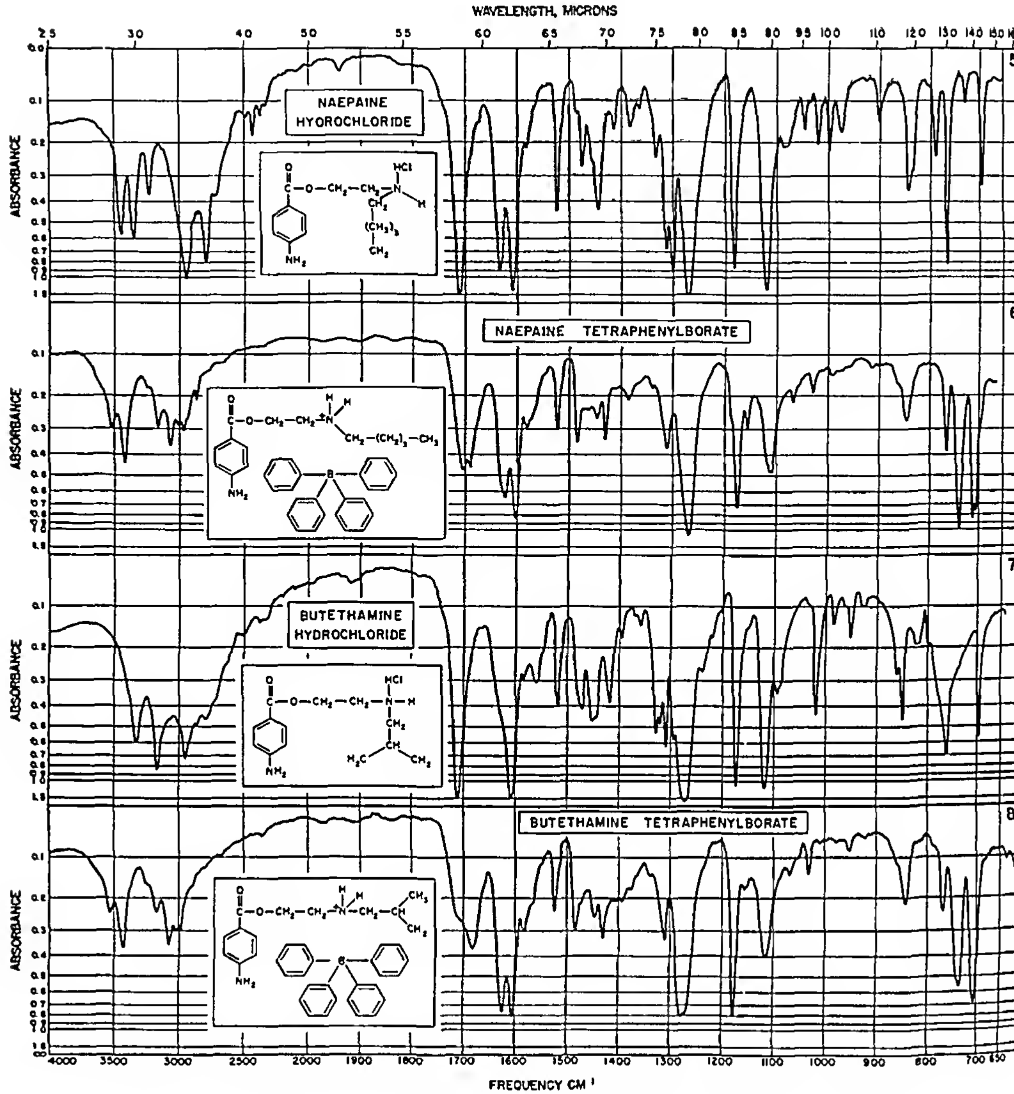

Fig 3 —Infrared spectra of some local anesthetics and their tetraphenylborates

$$[\text{RN H}]^+[\text{Cl}]^- + [\text{Na}]^+[(C_6H_5)_4\ \text{B}]^- \rightarrow [\text{RN H}]^+[(C_6H_5)_4\ \text{B}]^- + \text{NaCl}$$

The mineral acid salts of the organic bases generally display absorptions near 2,500 cm $^{-1}$ (N H stretching vibrations observed in amine hydrochlorides) (23) Their derivatives, on the other hand, exhibit no structure in this region which observation is in accord with the reaction mechanism illustrated in the equation shown The phenomenon is most pronounced in the spectra of tetracaine and procaine hydrochloride and their derivatives and least noticeable in the spectrum of butethamine hydrochloride and its tetraphenylborate

Further support for the process of salt formation is afforded by comparison of the spectra of piperocaine hydrochloride and its derivative The local anesthetic shows no structure in the 3,500–3,600 cm $^{-1}$ region, whereas a characteristic doublet in this wave length range is observed in the spectrum of the tetraphenylborate The absorption is to be ascribed to oscillations of the type of N H bond formed as a result of the reaction which establishes, generally, an electrostatic field of attraction between the organic bases and the tetraphenylboron moiety as shown in the structural formulas Infrared evidence thus illustrates how the vibration characteristics of the N H linkage present in the reactant are modified because of environmental effects in the derivative The phenomenon is not clearly recognizable in the spectra of the remaining tetraphenylborates included in this study because of the presence of both N HCl and N H groupings in the local anesthetics prior to reaction It should be noted in this connection that the absence of normal N H stretching bands in the chloroform spectra of piperidine and

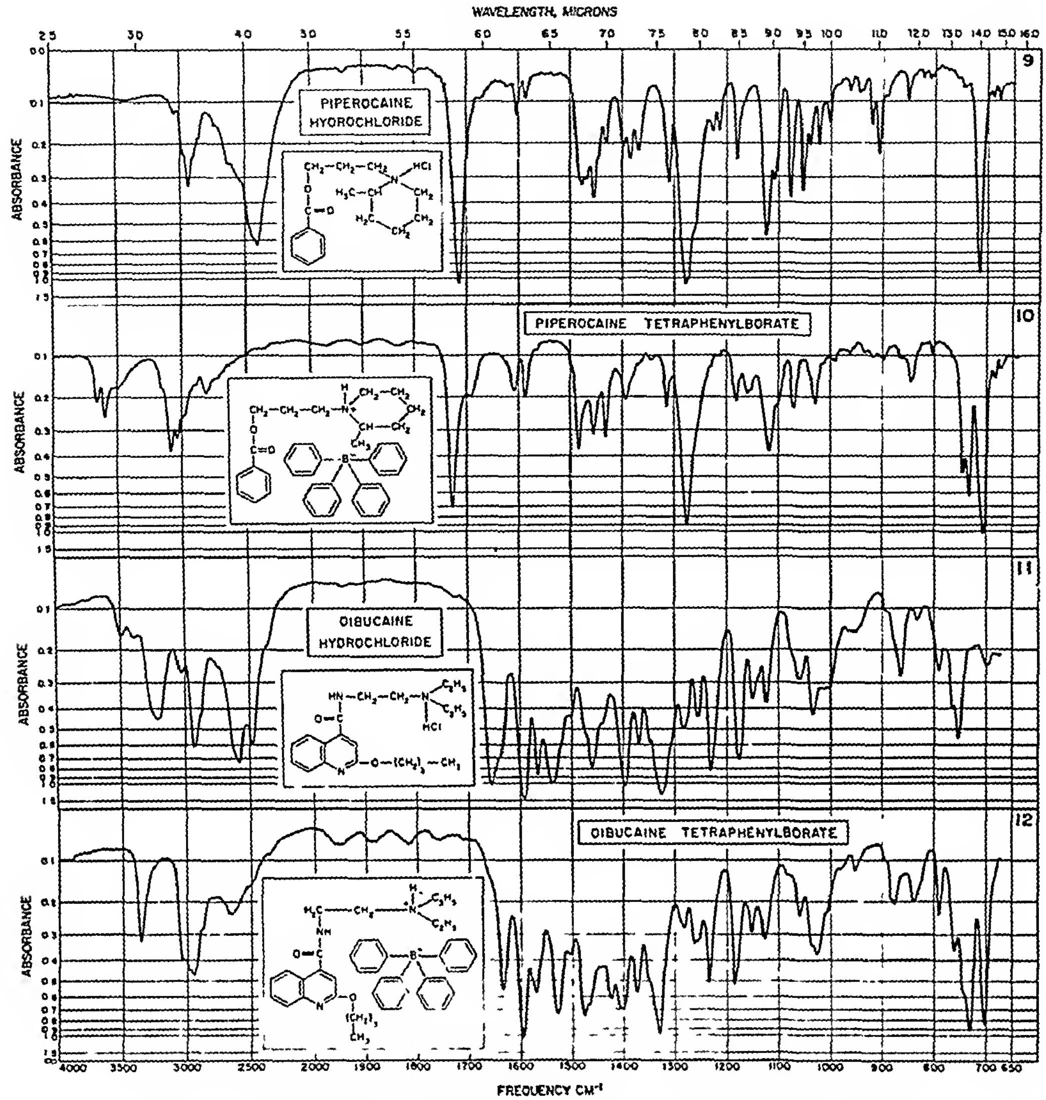

Fig. 3.—Infrared spectra of some local anesthetics and their tetraphenylborates.

some natural products containing this ring system was observed previously by Marion, Ramsay, and Jones (24). The phenomenon evidently also applies to the synthetic local anesthetic piperocaine (benzoic ester of γ-(2-methyl piperidino)-propanol) but is absent in the tetraphenylboron derivative.

The local anesthetics studied display several common characteristic structural features and, since these features are retained in the reaction product, the two series of spectra exhibit a number of common bands.

Characteristic N-H and C-H stretching absorptions are observed in the 3,500 and 3,000 cm.$^{-1}$ region, respectively. Strong bands also occur in the 1,700 cm.$^{-1}$ region because of the presence of a carbonyl group in these molecules. The absorption is both modified and displaced as a result of derivatization. In dibucaine hydrochloride it appears at 1,654 cm.$^{-1}$, which position is normal for secondary amines examined in the solid state. In the tetraphenylborate it is observed at 1,635 cm.$^{-1}$. In the spectrum of butethamine hydrochloride it appears as an intense, sharp band at 1,715 cm.$^{-1}$, whereas in the tetraphenylborate compound it is broadened and displaced toward lower frequency at 1,690 cm.$^{-1}$. In both procaine and piperocaine hydrochloride the band is intense, occurring at 1,700 and 1,718 cm.$^{-1}$, respectively. Their tetraphenylborates also display the absorption as a sharp peak but at shorter wavelengths (1,718 and 1,731 cm.$^{-1}$, respectively). In the spectra of tetracaine and nacpaine tetraphenylborate the band is split into a doublet. Since identical concentrations were used for the measurements the absorption is invariably more intense in the spectra of the local anesthetics than in the corresponding derivatives.

Intense absorption as either a single band or a doublet is observed in the 1,600 cm.$^{-1}$ region. It

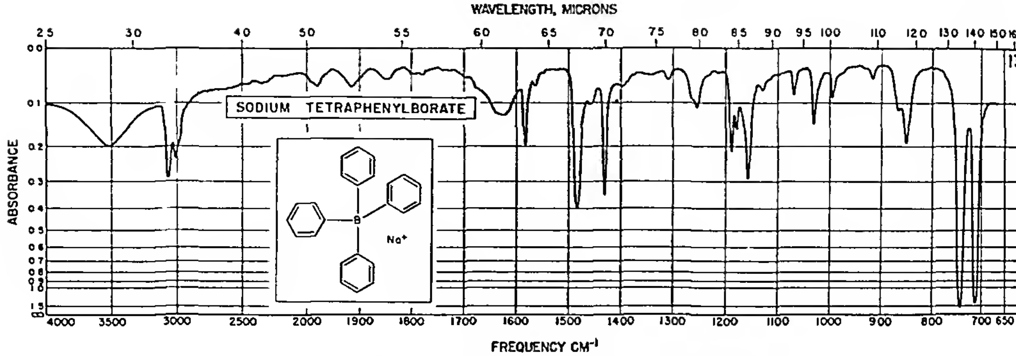

Fig 4 —Infrared spectrum of sodium tetraphenylboron.

TABLE IV —TITRATION OF TETRAPHENYLBORATES WITH PERCHLORIC ACID

Tetra-phenyl-borate	Equiva-lent	Taken, mg	Recovered, mg	Purity, %
Naepaine	M/2	101 6	100 1	98 5
		102 9	102 0	99 1
Dibueaine	M/2	102 2	101 2	99 0
		110 0	109 7	99 7
Piperoeaine	M	130 9	131 1	100 2
		116 2	116 0	99 8
Proeaine	M/2	101 0	100 0	99 0
		105 0	104 5	99 5
Tetraeaine	M/2	101 3	100 9	99 6
		102 2	101 7	99 5

TABLE V —ULTRAVIOLET CHARACTERISTICS OF SOME LOCAL ANESTHETICS AND THEIR TETRAPHENYLBORATES

Compound	λ Maximum, mμ	ε Max	λ Minimum, mμ	ε Min
Proeaine HCl	300	21680	245	885
Proeaine T P B	295	22315	240	4841
Piperoeaine HCl	274	3300	259	2365
Piperoeaine T P B	274	5261	256 5	4485
Naepaine HCl	299	20930	244	794
Naepaine T P B	295	22178	241	5576
Tetracaine HCl	314	25627	252	482
Tetracaine T P B	309	26244	246 5	4742
Dibucaine HCl	328	3760	297	1960
Dibucaine T P B	328	4527	300	3160
Butethamine HCl	299	21271	245	779
Butethamine T P B	295	22520	253	6672
Sodium T P B	266	3002	264	2853
	274	2271	272	2122

may be considered indicative of the deformation frequencies of the C_6H_5-N< bonds occurring in these molecules although its exact interpretation is complicated by the fact that aromatic ring vibrations also occur in this wavelength range Piperocaine hydrochloride and its derivative, which compounds lack the anilino structure, do not show this absorption.

Further intense bands are observed in the 1,275-, 1,175-, and 1,125-cm $^{-1}$ regions (C-N stretching vibrations of aromatic amines and C-O stretching vibration of aromatic esters). Only dibucaine hydrochloride, which is not an ester but an amide contain-

TABLE VI —POSITION OF KEY BANDS IN INFRARED SPECTRA OF LOCAL ANESTHETICS AND THEIR TETRAPHENYLBORATES IN THE 850-650 CM $^{-1}$ REGION

Compound	Characteristic Vibration Frequency, cm $^{-1}$	Intensity of Absorption
Tetracaine hydrochloride	834	s
	810	i
	765	vs
	740	m
	698	s
	648	m
Tetraeaine tetraphenylborate	831	m
	810, 803	Doublet
	770	s
	745	sh
	736	s
	708	s
	650	w
Proeaine hydrochloride	848	vs
	835–815	Triplet
	795	w
	770	vs
	736	w
	695	w
Procaine tetraphenylborate	840	s
	806	w
	784	vw
	773	w
	769	s
	745, 738	Doublet
	733	w
	705	vs
Naepaine hydrochloride	845	s
	837	vw
	792	s
	768	vs
	732	w
	695	vs
Naepaine tetraphenylborate	847	m
	767	s
	740	s
	712, 706	Doublet
Butethamine hydrochloride	850	s
	825	w
	800	i
	763	vs
	697	vs
Butethamine tetraphenylborate	845	m
	787	i
	770	m

TABLE VI (*cont.*)

Compound	Characteristic Vibration Frequency, cm⁻¹	Intensity of Absorption
Butethamine tetraphenylborate (cont)	743	i
	737	s
	708	vs
	635	vw
Piperocaine hydrochloride	850	w
	823	vw
	810	vw
	805	vw
	745	vw
	715	vs
	688	vw
	675	vw
Piperocaine tetraphenylborate	847	w
	805	vw
	743	Doublet
	735	
	707	vs
	685	vw
	675	vw
Dibucaine hydrochloride	833	w
	792	w
	763	vw
	753	s
	700	w
Dibucaine tetraphenylborate	840	m
	790	s
	762	w
	745	i
	732	vs
	703	vs

ing a quinoline structure, displays a different absorption pattern. The presence of the heterocyclic ring system is recognized by the four bands (two strong and two weak) observed in the 1,600–1,500-cm $^{-1}$ region (C = C and C = N stretching vibrations) and by the absorptions occurring at 865 and 736 cm $^{-1}$ (C — H deformation vibrations) In the tetraphenylborate derivative the 1,600–1,500-cm.$^{-1}$ profile remains unaltered but in the fingerprint region, which is more sensitive to total molecular structure, the absorptions are modified

In addition to the spectral features which reflect the presence of specific functional groups in both the parent compounds and their derivatives there are also found a number of characteristic bands which may be used to differentiate the various molecules from one another. Thus the reaction is of value to the forensic chemist and toxicologist In general, the spectra of the tetraphenylborates are richer in structure than those of the local anesthetics, particularly throughout the 850-650 cm $^{-1}$ region where spectral contributions of the reagent are found to be maximal This wavelength range is therefore most useful for identification purposes, as shown in Table VI which lists the characteristic absorptions occurring in this region

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In Vitro Test for Antiarrhythmic Agents*

By CHESTER W. DURACHTA†, and HUGH C. FERGUSON‡

A valid method for screening antiarrhythmic agents is described. Methods of producing and recording various types of arrhythmias are outlined, and response to standard drugs discussed.

THE MASS SCREENING of potentially useful medicinal agents should be rapid, reliable, and inexpensive Preliminary evaluation of a new substance should indicate whether additional experiments might be required or that further studies would be fruitless. The initial assay should be simple, calculations and technical manipulations kept at a minimum, and the results clearly defined and unequivocal If these conditions are satisfied the time saved could be used for additional screening, or active compounds could be subjected to more critical examination with the ultimate objective of clinical utility.

Methods for the production of auricular arrhythmias and ventricular fibrillation in the isolated rabbit heart have been described by Ferguson and Durachta (1). It was thought that this preparation might be used as a rapid screening

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method if the experimentally induced arrhythmias would respond favorably to clinically efficacious compounds such as quinidine sulfate, procainamide, and methacholine chloride. Therefore, studies were undertaken in order to determine the effects of these agents on the arrhythmias so produced.

EXPERIMENTAL

Methods.—Rabbits weighing 2.5–3.0 Kg. were sacrificed by a sharp blow on the head. The hearts were rapidly extirpated and set up as originally described by Langendorff (2) and modified by Locke and Rosenheim (3). Auricular flutters or paroxysmal tachycardias were induced electrically by stimulating the right auricle with 5–10 volts, square waves, at a pulse duration of 5–10 milliseconds, with a pulse delay of 5–10 milliseconds and a frequency of 50–150 cycles/second. If persistent auricular irregularities could not be instituted after ten to fifteen minutes, ventricular fibrillation was initiated by applying shocks of 100–140 volts, with no pulse delay, alternately and rapidly to both atria with a Grass stimulator. The latter technique was always immediately successful and spontaneous reversions of fibrillation were never observed. Auricular arrhythmias could also be produced by the topical application of 0.05% solution of aconitine nitrate. All compounds to be tested were prepared in Locke-Ringer's solution at a concentration of 200 µg./0.1 cc. Compounds were administered into the perfusate via a side arm cannula at geometrically progressive doses starting at 200 µg. Electrical potentials were recorded from lead II (right auricle-left ventricle) by means of a Twin Viso Sanborn Electrocardiograph (calibration: 1 millivolt = 5 or 10 mm., paper speed = 25 mm./sec.) at one, three, and five minutes after administering the compound to be tested, and if the arrhythmia persisted, the next higher dose was administered. This dosage schedule was continued until reversion to a normal sinus rhythm occurred or until a definite effect was produced.

RESULTS

In 49 experiments, persistent auricular flutters were initiated 22.5% of the time, paroxysmal tachycardias were obtained in 53.1% of the experiments, and ventricular fibrillations could always be produced. The results are summarized in Table I. When auricular arrhythmias could not be instituted compounds were screened for their effect against ventricular fibrillation.

TABLE I.—RESULTS OF EFFORTS TO PRODUCE PERSISTENT ARRHYTHMIAS IN THE ISOLATED RABBIT HEART

Arrhythmia Produced	Number of Trials	Number Successful	Successful %
Atrial flutter	49	11	22.5
Auricular paroxysmal tachycardia	49	26	53.1
Ventricular fibrillation	21	21	100.0

Quinidine Sulfate.—This compound was tried against auricular paroxysmal tachycardia, auricular flutter, and ventricular fibrillation and was active at an average dose of 0.431 ± 0.038 mg. (Table II). Figure 1 shows typical electrocardiographic potentials obtained before and during fibrillation and three minutes after the administration of 0.500 mg. of quinidine sulfate, at which time reversion to a normal sinus rhythm occurred. In all experiments, reversions to normal sinus rhythm were seen one to five minutes after administration of this agent.

Procainamide (Pronestyl).—This substance was tried on experimentally induced ventricular fibrillation and was active at an average dose of 1.58 ± 0.135 mg. (Table II). In all experiments reversions to normal sinus rhythm occurred one to five minutes after compound administration. Typical potentials seen during ventricular fibrillation are demonstrated by the electrograms in Fig. 2. In this experiment reversions to a normal sinus rhythm occurred approximately three minutes after administration of 1.6 mg. of procainamide.

Methacholine Chloride.—Mecholyl was active against auricular paroxysmal tachycardia at an average dose of 0.0043 mg. ± 0.0003 mg. (Table II), but was ineffective against atrial flutter and ventricular fibrillation. Auricular paroxysmal tachycardia is demonstrated by electrocardiographic potentials shown in Fig. 3. This irregularity was reverted to normal sinus rhythm one to two minutes

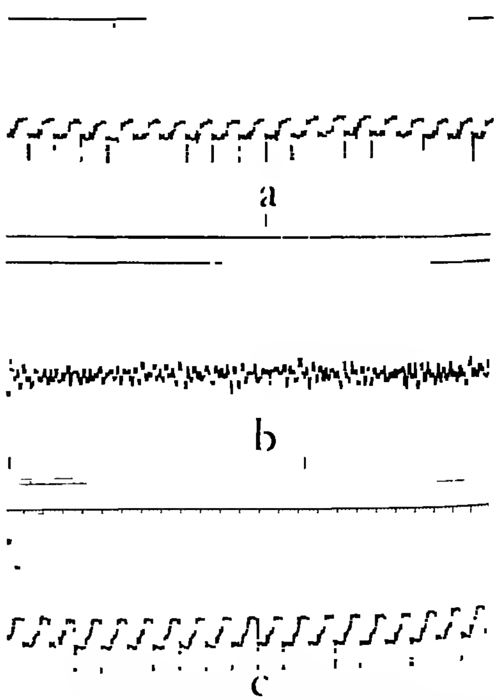

Fig. 1.—Electrocardiographic potentials obtained from the isolated rabbit heart. Calibration 1 mv. = 10 mm., paper speed = 25 mm./sec., lead II (= right auricle-left ventricle). (a) Pretreat record. Heart rate = 225 beats/minute. (b) Electrically induced fibrillation. (c) Record three minutes after administration of 0.500 mg. quinidine sulfate. Heart rate = 225 beats/minute.

TABLE II.—EFFECT OF VARIOUS ANTIARRHYTHMIC AGENTS ON ARRHYTHMIAS IN THE ISOLATED RABBIT HEART

	Quinidine Sulfate	Procainamide	Methacholine Chloride
Type(s) of irregularity induced	Auricular flutter Ventricular fibrillation Paroxysmal tachycardia	Fibrillation	Paroxysmal tachycardia
Number of trials	19	16	11
Successful reversions	19	16	11
Active dose (mg.)	$\bar{X}$ = 0 431 ±0 038	$\bar{X}$ = 1 580 ±0 135	$\bar{X}$ = 0 0043 ±0 0003

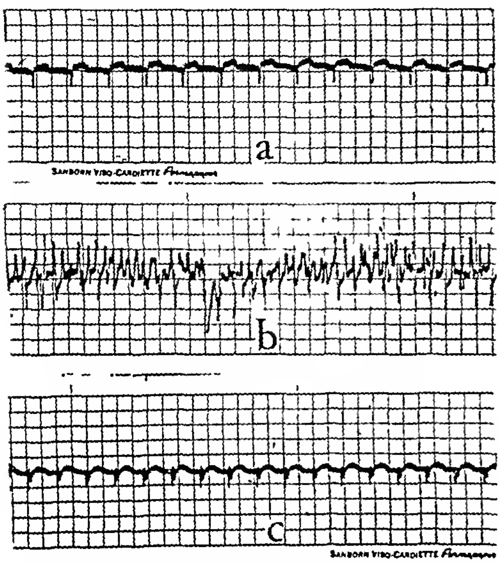

Fig. 2.—Electrocardiographic potentials obtained from the isolated rabbit heart. Calibration 1 mv. = 10 mm., paper speed = 25 mm./sec., lead II (= right auricle-left ventricle). (a) Pretreat. record. Heart rate = 90 beats/minute. (b) Electrically induced fibrillation. (c) Record three minutes after administration of 1.6 mg. procainamide. Heart rate = 120 beats/minute.

after the administration of 0.001 mg. of methacholine chloride.

In conditions other than auricular paroxysmal tachycardia, bradycardia or complete arrest occurred following larger doses of methacholine chloride; upon resumption of cardiac activity no remission of auricular flutter or ventricular fibrillation was seen.

DISCUSSION

Our results demonstrate that the Langendorff heart can be easily modified for use as a screening tool for antiarrhythmic agents Clinically efficacious compounds such as quinidine sulfate, procainamide (Pronestyl), and methacholine chloride have produced remissions of cardiac irregularities in the isolated rabbit heart which are similar to those found in human cardiac patients The quantity of material needed is very small and this lends itself well to screening programs where newly synthesized compounds are not generally in great supply Unfortunately, water insoluble compounds cannot reliably be tested by this method and must be tried on intact preparations as those employed by Rosenbleuth and Garcia Ramos (4, 5), Scherf (6), and Harris (7) It has been shown that, while auricular flutter and

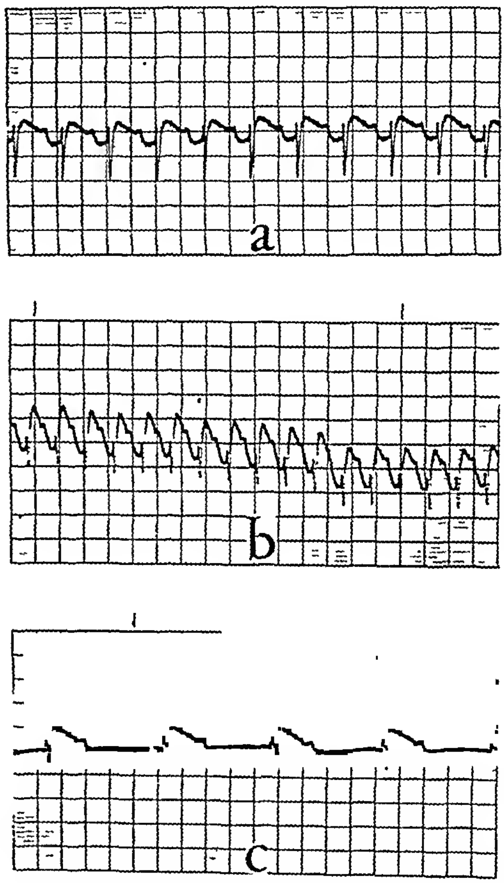

Fig. 3.—Electrocardiographic potentials obtained from the isolated rabbit heart. Calibration 1 mv. = 10 mm., paper speed = 25 mm./sec., lead II (= right auricle-left ventricle). (a) Pretreat. record. Heart rate = 142 beats/minute. (b) Electrically induced paroxysmal tachycardia. (c) Record two minutes after administration of 0.1 ml. of 1:100,000 methacholine chloride (0.001 mg.). Heart rate = 48 beats/minute.

paroxysmal tachycardia cannot always be instituted• the preparation need not be discarded since ventricular fibrillation can always be initiated by appropriate electrical stimulation Determination of the average active dose of standard compounds such as quinidine sulfate, procainamide, or methacholine chloride reveals the relative potency of unknown substances, and determination of the LD_{50} permits an estimate of a compound's margin of safety. Ultimately, knowledge of these factors assists in choosing the safest and most active agents.

SUMMARY

Methods for producing various types of arrhythmia in the isolated rabbit heart have previously been presented These methods were thought to be potentially useful in screening for antiarrhythmic agents if the irregularities would respond favorably to clinically efficacious agents Quinidine sulfate, procainamide, and methacholine chloride were tried and found to be consistently active at low doses It has been our experience that this is a rapid, reliable, and inexpensive method for screening antiarrhythmic agents

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Microestimation of Opium Alkaloids in Pharmaceuticals by Paper Chromatography*

By KLAUS GENEST and CHARLES G. FARMILO

A method of analysis for morphine, codeine, thebaine, and papaverine in mixtures employing paper chromatographic separation followed by densitometric evaluation with a self-integrating densitometer has been developed. The method has been applied to a number of pharmaceuticals and the results compared with those of other methods.

THE NEED for improvement of existing methods for identification and quantation of opium alkaloids was pointed out in a recent United Nations report (1a) Genest and Farmilo (1b) recently reviewed paper chromatographic analysis of narcotics and found only a few publications which deal with quantitative analysis of opiates Svendsen (2) estimated morphine in opium by means of a colorimetric method after elution from the chromatogram Svendsen, Aarnes, and Paulsen (3) compared this method with a densitometric method in which the density of the morphine spot (Folin-Ciocalteu reagent) is measured directly on the paper Asahina and Ono (4–7) estimated five principal opium alkaloids directly on the paper chromatogram by measuring the maximum absorbance in the U V region Schultz and Strauss (8) applied manual planimetry of chromatograms stained with a ferrichloride ferricyanide reagent for the estimation of morphine in opium tablets Paerregaard (9) determined morphine in urine by polarography of the morphine eluted from the chromatogram Miram and Pfeifer (10) reported a chromatographic method for the analysis of pure solutions of codeine and papaverine requiring the production of a contact negative copy of the chromatogram which was subsequently scanned with the photometer This step was followed by planimetry of the densitometric trace of the copy to obtain standard area *vs* concentration graphs Holubek, Kudrnáč, and Novák (11) estimated codeine and narcotine in poppy capsules after paper chromatographic separation by colorimetry and polarography, respectively

It is the object of this publication to describe a quantitative paper chromatographic method for opiate analysis which avoids elution, photographic reproduction, or manual planimetry by use of a self integrating densitometer

EXPERIMENTAL

Apparatus and Materials—The chromatography was conducted by a descending technique on Whatman No 1 paper strips, one inch wide, using cylindrical glass tanks (12 in x 24 in) Ten strips were run in each tank at one time Details on the salting of the paper with $(NH_4)_2SO_4$ (2%), the preparation of the solvent (iso BuOH AcOH H_2O 10 1 2 4) and the spray reagent (potassium iodoplatinate) and other chromatographic conditions are the same as those that have been described previously (12, 13), for an ascending technique

The alkaloid solutions to be estimated were spotted by means of an "Agla"-micrometer syringe Five λ of a test solution were applied per strip yielding a spot not larger than 5 mm in diameter The distance from the solvent to the origin of the chromatogram was 8 cm

The Spinco "Analytrol" Model RA[1] with focused tungsten lamp, 1½ mm aperture and B 2 cam (absorbance) was used No filter was employed in the final procedure The time for scanning of a chromatogram of 40 cm length containing four spots was approximately three minutes

PROCEDURE

Preparation of Standard Curve—Standard solutions of each of the four alkaloids, morphine, co

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[1] See Beckman/Spinco Bulletin RIM 2 for description and operation of Analytrol

deine, thebaine, and papaverine, containing from 1 to 10γ/λ in methanol, were prepared. After spotting 5λ of this solution on the strips they were equilibrated with the aqueous phase of the solvent for six hours, after which time the mobile phase was poured into the jar through a separatory funnel. Development required sixteen hours (overnight), in which time the solvent front traveled about 38 cm. After drying, the chromatograms were thoroughly sprayed on both sides with the potassium iodoplatinate reagent and dried again for fifteen minutes in a current of air prior to scanning in the densitometer. The densitometer was calibrated each time another spot was measured. This was done by means of a neutral density filter using the area of the strip containing only chromogenic agent immediately in front of each stained spot. Twenty to thirty strips (in batches of five each) were chromatographed for each concentration of each alkaloid. Standard curves prepared from these data can be seen in Fig. 1.

Application to Pharmaceuticals.—One practical application of the method is demonstrated by using nine pharmaceuticals containing one or more opium alkaloids. The extraction of tablets was made by shaking the powdered material mechanically for half an hour with water. In case of free bases, or slightly soluble alkaloidal salts the extraction was made with glacial acetic acid and diluted with water to a final concentration of 50% acetic acid. The extract was centrifuged and an aliquot of liquid containing between 10 and 30γ of the alkaloid was spotted.

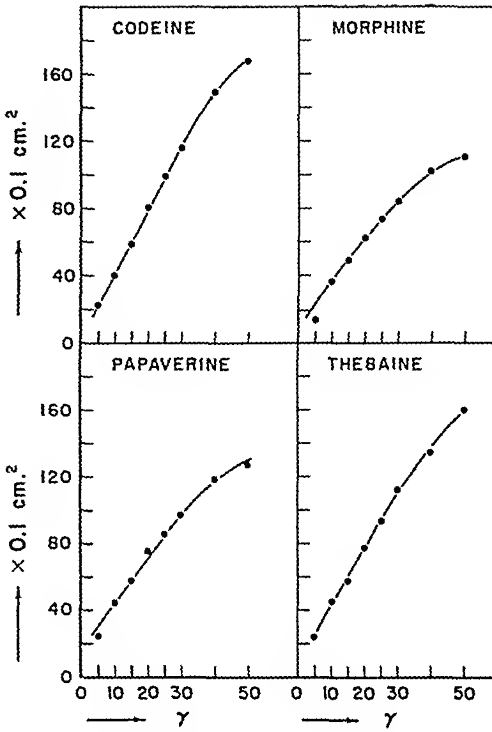

Fig. 1.—Standard curves of four opium alkaloids.

RESULTS AND DISCUSSIONS

Background Color.—The background color of the chromatograms presents a critical problem influencing the simplicity and reproducibility of this method. Considerable investigation of the details of the effect of the variables controlling this color was made. Salting of the papers with phosphates, citrates, or acetates gave brown to pink background colors after spraying. These colors gradually fade to a yellowish shade The light beam of the densitometer could not penetrate these chromatograms without the use of agents which improve transparency (glycerol, bromo-naphthalene in paraffin oil, varnish, etc.). Salting of the paper with $(NH_4)_2SO_4$ proved to be preferable since it fulfills a dual purpose: round spots without trailing and good separations are obtained, and a background of slightly gray to pinkish color is produced which is easily penetratable by the light ray. Although this combination of salting and chromogenic agent is the choice condition for quantation there are small differences in absolute background density of different chromatograms. To examine the influence of these changes of the absolute density of the background on the reproducibility of the results, artificial backgrounds were created in the following way: A spot of methyl violet on a Whatman No. 1 strip was scanned both with and without sheets of transparent paper between the background of the strip and the scanning photocell. The absorbance (expressed in mm.) of the combined chromatogram plus transparent paper was then recorded. The densitometer chart recording pen was then reset to the zero line, thereby suppressing the enhanced background and the spot was scanned again. The results shown in Table I indicate that suppression of the background up to 19 mm. has little influence on the total absorbance of the methyl violet spot. In replicate chromatograms, the difference in background readings was within ±5 mm.

Cams and Filters.—Experiments to demonstrate the effect of scanning the same chromatograms with different cams and filters were carried out. The results can be seen in Table II. The difference in shade of the spots of the four alkaloids becomes evident by the dissimilar relative response when scanned with and without the filter. Scanning with a blue filter gives a higher total absorbance than is obtained without the filter. However, the irregularities of the background color combined with the texture of the paper were too marked to allow a proper baseline adjustment close to the zero line which militated against its use. Irregularities of background were still more evident when using the densitometer B-1 cam along with other filters. The absorbances were mostly out of range of the instrument for the 35γ level and were not reproducible. Densitometer cam B-2 without a filter was therefore chosen as the equipment for the quantation of opiate chromatograms.

Machine Error.—A spot of red ink on a Whatman No. 1 strip and a morphine spot on a chromatogram were scanned ten times each to estimate the error of replicate scanning. The standard deviations were found to be ±1.45% and ±0.75%, respectively.

Stability of Color.—The alkaloid iodoplatinate stained spots of the four opiates were quite stable after spraying for periods from fifteen minutes to six

TABLE I.—MEASUREMENT OF CHANGES IN BACKGROUND DENSITY BY ANALYTROL

Background Adjustment	Total Color Density (Area Under Curve in 0 1 cm ²)			Average
Normal	135	137	136	136 0
Suppression, 6 5 mm	138	135	136	136 4
Suppression, 19 0 mm.	138	137	138	137 7

TABLE II.—SCANNING OF A PAPER CHROMATOGRAM WITH DIFFERENT CAMS AND WITH AND WITHOUT BLUE FILTER[a]

	Cam No			
Alkaloid[b]	B_2, no filter	B_2, filter	B_3, no filter	B_3 filter
Papaverine	64	100	29	51
Thebaine	97	128	50	74
Codeine	108	122	66	82
Morphine	83	83	50	48

[a] Corning 5031
[b] 30γ of each alkaloid

hours Results of the color stability study are given in Table III If chromatograms cannot be scanned immediately after drying (15 min) care should be taken to protect them from daylight Three strips stored in the dark were rescanned after three months and showed an average decrease in absorbance for papaverine, thebaine, codeine, and morphine of 9, 7, 3, and 2%, respectively

Standard Curves.—Figure 1 shows the standard curves of four opiates The plotted points represent the average of 20 estimations for each concentration of alkaloid. The experiments to obtain these results were made on consecutive days at room temperature (26° ± 1°), in one chromatographic tank using the same lot of salted paper for each experiment The average of the standard deviations of 640 experiments was ±3 94% The standard curves show a linear concentration *vs* total density relationship from 5γ to approximately 30–40γ. The best reproducibility was found between 10 and 30γ Figure 2 shows a reproduction of an original chromatogram including the densitometer trace The jagged lines between the spots

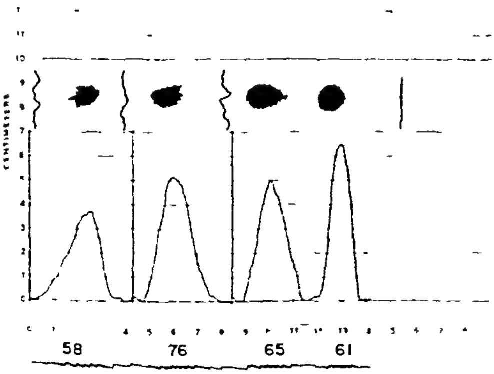

Fig 2 —Chromatogram of four opium alkaloids and evaluation by "Analytrol."

TABLE III.—STABILITY OF COLOR

Time, min	Total Color Density (Area Under Curve in 0 1 cm ²) Papaverine[a]	Thebaine[b]	Codeine[a]	Morphine[a]
15	71	92	110	60
30	71	91	104	60
40	66	87	102	59
55	66	88	101	61
65	67	88	102	58
120	68	92	104	59
240	66	87	100	60
360	63	87	103	60
Next morning (22 hr)	64	84	101	62

[a] 20γ [b] 25γ

in Fig 2 indicate where the original chromatogram was cut for mounting above the maximum of the densitometer trace. (R_f values for morphine, codeine, thebaine, and papaverine on this chromatogram were found to be 0 09, 0 18, 0.56, and 0 72, respectively.) Mounted below the spots in Fig. 2 are the bell-shaped tracings of absorbance of the alkaloidal spots The serrated line, at the bottom of Fig 2 represents the tracing of the densitometer integrating pen. The area under the curve corresponds to the total color density since the B-2 cam is calibrated in terms of absorbance One tooth of the serrated trace corresponds to 0 1 cm.².

Application to Pharmaceuticals.—The paper chromatographic method was applied to a variety of pharmaceuticals which contained, in addition to opium alkaloids, a number of other active ingredients as shown in Table IV. All analytical values are averages of duplicates. The results in Table IV indicate agreement between the values obtained by the chromatographic method and the methods of comparative assay

The sets of experiments to reproduce the standard curves were repeated at intervals It was found that the calibration graphs were sometimes not reproducible within the standard deviation

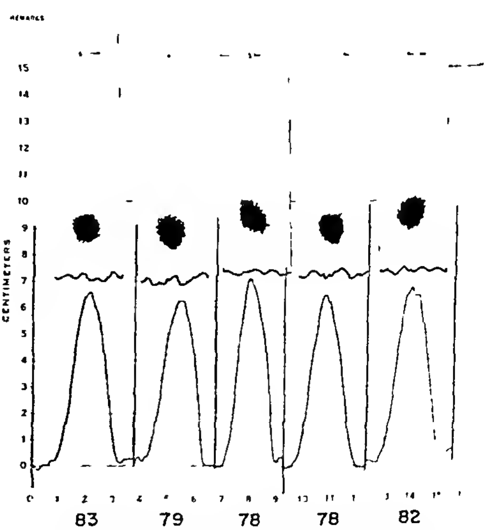

Fig 3 —Replicate analysis of APC and C tablets

TABLE IV.—PAPER CHROMATOGRAPHIC AND COMPARATIVE ANALYSIS OF PHARMACEUTICALS

Drug	Opium Alkaloids	Other Active Ingredients	Medium of Extraction	Labeled Strength, mg.	Densitometric Assay, mg. By Standard Curve, mg.	Densitometric Assay, mg. By Comparison,[a] mg.	Comparative Assay, mg.	Method for Comparative Assay
A	Papaverine	Nicotinic acid, nicotinamide	Acetic acid	80	80	...	80.7	$CHCl_3$-Shake out, Reineckate (14)
B	Papaverine·HCl	None	Acetic acid	15	15.3	14.9	14.9	UN-K/34 (15)
	codeine phosphate			15.	15.7	16.4	15.2	
C	Morphine HCl	Tinct. chloroformi et morphinae co.	None (dilution with ethanol)	(1%)	(0.96%)		(0.99%)	BPC 1954
					(1.07%)	(1.06%)		
D	Codeine phosphate	APC[b]	H_2O	32	32.6	31.6	32.4	Nonaqueous titration (16, 17)
E	Codeine phosphate	APCC[c]	H_2O	8	8.7	8.9	8.4	Nonaqueous titration (16, 17)
F	Codeine phosphate	APCC[c]	H_2O	16	15.3	15.3	16.3	Nonaqueous titration (16, 17)
G	Codeine phosphate	APCC[c]	H_2O	32	30.8	30.6	31.7	Nonaqueous titration (16, 17)
			acetic	32	33.3	34.7	...	
H	Morphine sulphate	None	H_2O	16.2	18.3	16.9	17.0	BP (1948)
I	Morphine sulphate	None	H_2O	10.8	11.4	11.4	11.1	BP (1948)

[a] Value obtained by comparison with standard reference run simultaneously.
[b] Aspirin, phenacetin, and caffeine
[c] Aspirin, phenacetin and caffeine citrate

(±3.94%) when the conditions of chromatography were altered, e. g., different jars, lots of salted papers, humidity, and temperature. Therefore, it is recommended that a standard concentration of the known alkaloids be included within each experiment.

One advantage of this chromatographic method is that in the preparation of the samples, no elaborate extraction procedure with organic solvents is required. Tinc. chloroformis et morphinae co. preparation could be used directly. Other water extractable ingredients in these preparations did not interfere with the procedure. An example of a replicate analysis of an assay of an aspirin, phenacetin, caffeine, and codeine tablet (one-half grain of codeine phosphate per tablet) is given in Fig. 3. One excipient, a yellow dye, was found close to the point of origin of the chromatogram which is not visible in the photographic reproduction of Fig. 3.

SUMMARY AND CONCLUSIONS

The densitometric paper chromatographic procedure was applied to pharmaceutical preparations containing micro quantities (5–50 γ) of alkaloids with an average standard deviation of ±3.94 per cent. A simple preliminary sample preparation step is required. The use of a self-integrating densitometer enables the quantation to be achieved more rapidly than by usual procedures involving elution, etc. The procedure is applicable to a wide range of alkaloids and synthetic basic compounds. The type of compound affects the slope of the calibration curve. Application of the quantitative paper chromatographic method to the assay of small samples of narcotics received in legal cases and to the analysis of opium for purposes of determination of country of origin gave satisfactory results with the expenditure of a minimum of labor, time, and material.

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The Biological Action of Cellular Depressants and Stimulants I*

The Action of Ethyl Carbamate (Urethane) on the Endogenous Respiration and the Rate of Cell Division in *Tetrahymena pyriformis*

By JOHN J. EILER, JOSEPH Z. KREZANOSKI†, and KWAN-HUA LEE

The effect of urethane on the endogenous respiration and on the rate of cell multiplication has been studied in normal and in trained cells of *Tetrahymena pyriformis* with the view of obtaining information regarding the mode of action of narcotics.

The classical view (1) that narcotics exert their characteristic biological effect through *inhibition of cellular respiration persists at this* time as one of several working hypotheses (2, 3). Despite the excellent studies by Quastel (4) and his associates, it has not been established that a depression of the respiratory activity in an oxygen-consuming cell is a primary, or even a necessary, event in the biological action of the narcotic or depressant drugs.

In studies on several classes of unicellular organisms, Fisher and co-workers (5–8) have shown that narcotics cause almost complete cessation of cell division at concentrations which depress cellular respiration only moderately. In studies on *Tetrahymena*, Ormsbee and Fisher (6) showed that urethane caused almost complete inhibition of cell multiplication at a concentration (0.1 *M*) which inhibited the endogenous respiration only about 25 per cent. Further, the regression line relating the rate of the endogenous respiration to an extended range of drug concentrations was such as to suggest the existence of two parallel resiratory systems with the more drug-sensitive system being geared to the endergonic processes of cell multiplication. Numerous studies (9–11) on a variety of biological forms are compatible with the view that growth and other energy-requiring processes may be geared to a specific fraction of the total respiration. Indeed, recent studies (12) on rat liver mitochondria suggest alternate pathways of electron transport, only one of which is phosphorylative and work promoting.

The successes which have been achieved (13, 14) in training organisms to grow in the presence of otherwise inhibitory concentrations of a drug suggested to us a possible means of putting to test the relationship suggested by Ormsbee and Fisher (6). Considerable support would be gained for the proposed gearing if, for example, organisms trained to grow in otherwise inhibitory concentrations of drug showed a concomitant decrease in sensitivity of a specific fraction of the respiration toward the depressing effects of the drug. Related to the question of the gearing between respiration and an endergonic process is the problem of whether the primary site of action of the drug is on respiration or on the endergonic process (3). A given set of results obtained in a training experiment, as just cited, could shed light on this vexing problem as well.

In the experiments reported here, we have studied the effects of urethane (ethyl carbamate) on the rate of cell multiplication and on the endogenous respiration in trained and untrained cultures of *Tetrahymena pyriformis.*

EXPERIMENTAL AND RESULTS

Materials and Methods.—The W strain of *Tetrahymena pyriformis* used in this study was graciously supplied by Prof. Daniel Mazia. Stock cultures were maintained in 300-ml. Erlenmeyer flasks containing 40 ml. of a medium consisting of 1.8% proteose-peptone (Difco) and 0.2% yeast extract (Difco). The cultures were maintained at 27° in the dark with weekly transfers. The sterility of both the stock and the experimental cultures was checked by microscopic observation and by plating on agar. Urethane was added to the medium as a sterile solution prior to inoculation. The appropriate concentration was sterilized by passage through a bacterial filter.

The turbidity measurements were made with a Klett-Summerson photoelectric colorimeter, using the No 42 filter to reduce the influence of the colored medium. All turbidity values are reported in Klett units

The procedure used for the counting of cells was essentially that of Hall, Johnson, and Loefer (15), using a Sedgewick-Rafter counting chamber (Scientific Glass Co , No. W4000) and a Whipple ocular micrometer Cells were killed through addition of an aliquot of diluted formalin to yield a final concentration of 1% formaldehyde. When necessary, samples were diluted with a 0.21% solution of sodium chloride. The total number of cells counted in

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each sample was between 500 and 1,000. Recently Scherbaum (16) has established that the error in this counting procedure is about 5%.

The alkali-soluble biuret-detectable protein was determined on washed cell preparations using the procedure of Gornall, *et al.* (17). Cells were collected and washed with the above-mentioned solution of sodium chloride through the use of centrifugation at 2,500 r. p. m. (International Centrifuge, size 1) for five minutes. The biuret reagent was added directly to the cells washed free of medium and the absorbance was determined in a model B Beckman Spectrophotometer, using crystalline bovine albumin as a standard. The values so obtained were the same as when the washed cells were first treated with trichloroacetic acid and the precipitated protein washed with diluted trichloroacetic acid prior to the addition of the biuret reagent. The simpler of the two procedures was used since all values are comparative and not absolute.

Growth Studies.—All critical growth trials were carried out using 2,000-ml. Povitsky flasks containing 750 ml. of culture medium. The reasonably large surface-to-volume ratio thus obtained fostered rapid growth and the large volume permitted the frequent withdrawal of samples without marked changes in the surface-volume ratio of the culture. Further, in this fashion, the growth studies were conducted under the same conditions as were necessary to obtain sufficient cells for the respiration studies.

First, the effect of various concentrations of urethane on the rate of increase of cell mass was determined in order to insure that our strain of *Tetrahymena* behaved similarly to the one used by Ormsbee and Fisher (6). The results of such growth trials testing the effect of six concentrations of urethane are presented in Fig. 1. In these trials each flask, containing 750 ml. either of the medium or the medium with the indicated concentration of urethane, was inoculated with 5.0 ml. of a suspension (5×10^5 cells) from a stock culture in the rapid phase of growth. The flasks were incubated at 27° in the dark. Aliquots were withdrawn about every twelve hours for the determination of turbidity and cell protein. Only the results of the protein determinations are given in Fig. 1 since the Klett's units and the protein content were directly related throughout all growth trials with and without the several concentrations of urethane. The results, showing a graded effect of the several concentrations of the drug with essentially no growth at 0.11 *M*, are in good agreement with the findings of Ormsbee and Fisher (6).

To test the possibility of training the protozoa to overcome, at least partially, the growth-inhibiting effects of urethane, the organisms were subcultured in Klett-Summerson tubes containing 5.0 ml. of either 2.0% proteose-peptone (Difco) or the same medium with urethane. The 0.1 ml. of inoculum was taken from a proteose-peptone culture in the stationary phase of growth. The tubes were incubated in the dark at 25°. Two-hundredths *M* urethane was selected as the concentration for initial exposure of the cells to undergo training, since at this concentration growth is only slightly inhibited or even stimulated. Both normal cells and the cells undergoing training were subcultured every five days. The cells under training were transferred

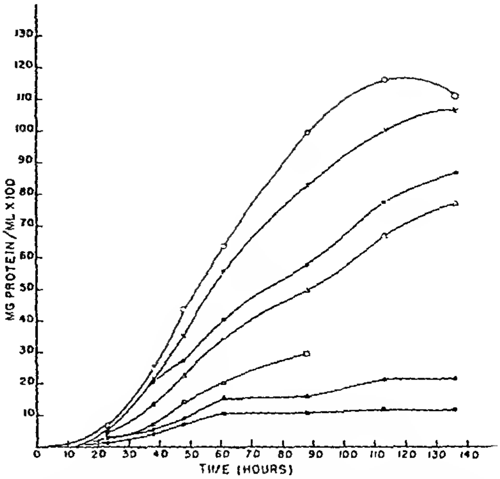

Fig. 1.—The effect of several concentrations of urethane on the growth of normal cultures: O = control; X = 0.03 *M*; ● = 0.056 *M*; Δ = 0.07 *M*; □ = 0.09 *M*; ▲ = 0.10 *M*, and ■ = 0.11 *M*. The ordinate represents the experimental value times 100.

twice into medium containing 0.02 *M* urethane and twice into medium containing 0.04 *M* drug. The normal or control cells were subcultured with the same frequency.

To test the results of training, the time-dependent increases in turbidity of the normal cells in the presence and in the absence of 0.08 *M* urethane were compared with the increases of the drug-exposed cells in the presence of 0.04 *M* and 0.08 *M* urethane. The results presented in Fig. 2 indicate that the cells trained in 0.04 *M* drug increased in mass at about the same rate in the presence of either 0.04 *M* or 0.08 *M* urethane as did the normal cells in the absence of the drug. The cells previously unexposed to the drug showed a greatly retarded rate of increase of mass in the presence of the 0.08 *M* urethane. Clearly, training under these conditions leads to an almost complete reduction in the sensitivity to the growth-inhibiting effects of 0.08 *M* urethane. The pronounced lag in growth observed in all trials in Fig. 2 is due to the use of mature cultures as inocula in the subculturing and in the testing, and is not due to the action of the drug.

Similar studies indicated that concentrations above 0.08 *M* urethane could not be used for testing. When the training concentration was raised to 0.1 *M* only meager growth was observed for several subculturings, followed by complete failure of growth.

It was necessary to alter the conditions for training to provide sufficient uniform material for the later studies. All subsequent subculturing made use of 300-ml. Erlenmeyer flasks containing 40 ml. of medium with or without urethane. To permit more rapid growth than that indicated in Fig. 2, the medium consisted of 1.8 proteose-peptone and 0.2% yeast extract, as in the stock cultures.

Trials testing the effectiveness of training under the new conditions were carried out using turbidity as the index of cell mass. The results of one set of trials are presented in Fig. 3. The cells undergoing training were brought through a series of concentra-

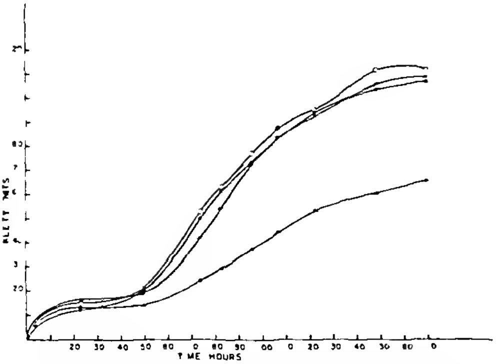

Fig 2—The effect of urethane on the growth of test tube cultures of trained and untrained cells ○ = control, ● = trained in 0 04 M urethane and tested in 0 04 M, X = trained in 0 04 M urethane and tested in 0 08 M, ▲ = untrained and tested in 0 08 M

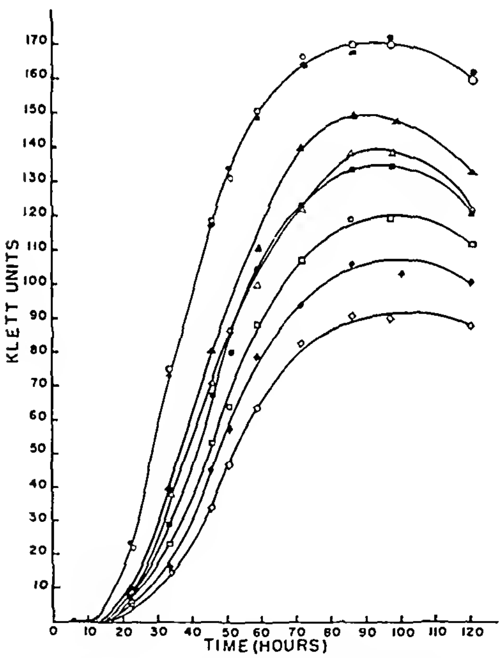

Fig 3—The effect of urethane on trained and untrained cells which had been cultured in Erlenmeyer flasks containing the standard medium Normal cells, ○ = control, △ = 0 07 M, □ = 0 08 M, and ◇ = 0 09 M Trained cells, ● = control, ▲ = 0 07 M, ■ = 0 08 M, and ◆ = 0 09 M

tions of urethane and, at the time of the growth trials, had been subcultured in 0 08 M urethane every forty-eight hours for four times (two hundred sixteen hours) Control cells were subcultured in the plain medium with the same frequency The growth trials were carried out in 300 ml Erlenmeyer flasks fitted with a side arm suitable for insertion into the Klett Summerson instrument Forty ml of medium or medium with urethane were inoculated with 0 5 ml of suspension from either of the two final subcultures,

The results given in Fig 3 indicate that subculturing and testing under conditions which permit greater oxygenation (surface-volume ratio) and more rapid growth do not give rise to so great a decrease in sensitivity to the drug as was observed in the results presented in Fig 2 All three test concentrations of urethane produced a proportional decrease in the rate of increase in cell mass However, there remains a significant effect of training, since in each test concentration the drug exposed cells multiplied (turbidity) more rapidly than the previously unexposed cells

Similar studies demonstrated that shorter periods of training in 0 08 M urethane produced no measurable increase in drug resistance Extension of the training period, with frequent subculturings, during fifteen days, or even six months, did not give better results than those presented in Fig 3 In no case did training permit growth in the presence of 0 1 M urethane

To observe the effect of training on increase in cell mass as judged both by turbidity and cell count, growth trials were conducted in Povitsky flasks The 750 ml of medium with or without urethane were inoculated with either trained or normal cells to yield an initial density of about 1,000 cells per ml The trained cells had been subcultured in 0 08 M urethane every forty eight hours for fifteen days The normal cells were subcultured with the same frequency Samples were withdrawn approximately every twelve hours during the incubation period

To conserve space, the results of the influence of the drug on the turbidity of the cultures are presented in Fig 4 only as a regression line correlating turbidity with cell count The cell count and Klett reading for every sample taken from both the trained and untrained cell cultures were used in the preparation of Fig 4 While several factors undoubtedly contribute to the observed lack of linearity in the regression line, the principal factor relates to dead cells and debris As the cultures increase in age, the number of dead cells and the debris contribute to the Klett readings in a manner not reflected in the cell counts which included principally live cells It is significant to point out that the results from both types of cells fit the curve equally well This fact precludes the need to present both cell count and turbidity data and suggests (18) that there is no great disparity in size between the normal and the trained cells Closer attention was not given to the effect of training on cell size and morphology

The results of the effect of the drug on the rate of increase in cell count are presented in Fig 5 as a semilog curve, wherein the time dependent count divided by the initial count is plotted on a logarithmic scale against time It is clear from Fig 5 that both the trained and the untrained cells multiplied at an almost identical rate in the absence of drug, while the trained cells multiplied more rapidly in each of the test concentrations of drug Thus, training produced an increase in drug resistance as judged both by turbidity (cell mass) and cell count The lack of linearity which develops at about the fifth generation is related to oxygen want and will be considered more fully in the second paper in this series (19) The period of logarithmic growth, as is

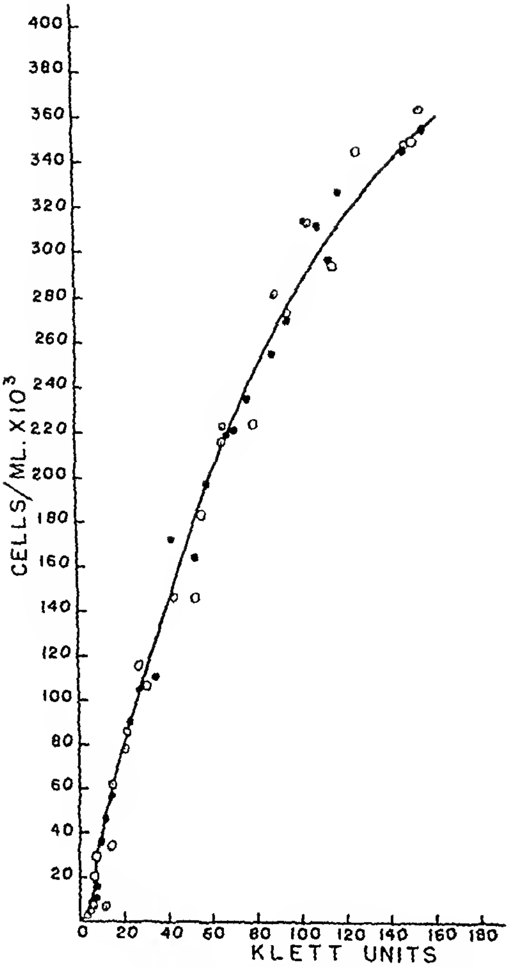

Fig. 4.—Correlation of cell numbers with Klett readings. Open circles relate to normal cells; solid circles relate to trained cells. The values on the ordinate are to be multiplied by 1,000 to yield the experimental value.

customary, has been used to calculate the mean generation time, as given in Table I, to provide quantitative estimates of the effect of training. The values for the per cent increase in mean generation time presented in Table I indicate that each of the test concentrations have about one-half the inhibitory effect on the trained cells as upon the untrained cells.

Respiration Studies.—The effect of various concentrations of urethane on the rate of the endogenous respiration of normal and trained cells was measured at 27° essentially according to the procedure of Ormsbee and Fisher, using the conventional Warburg technique. The drug-exposed cells had been subjected to weekly subculturing in 0.08 *M* urethane for the period of approximately six months. To obtain sufficient cells, the final growth was conducted in Povitsky flasks. The normal cells were harvested from the 750 ml. of medium at the end of forty-eight hours while the trained cells were harvested from the 750 ml. of urethane-containing medium at the end of seventy-two hours. In this

TABLE I.—THE EFFECT OF ETHYL CARBAMATE ON THE GENERATION TIME OF NORMAL AND TRAINED *Tetrahymena*

Cell Type	Molar Concn. of Urethane	Mean Generation Time[a] Time, Hr.	Increase, %
Normal	0.00	3.4	..
	0.07	4.4	29
	0.08	5.0	47
	0.09	5.6	65
Trained	0.00	3.4	..
	0.07	4.0	18
	0.08	4.3	26
	0.09	4.7	38

[a] The mean generation times were calculated from the first-order rate constants estimated from the slopes of the curves in Fig. 5.

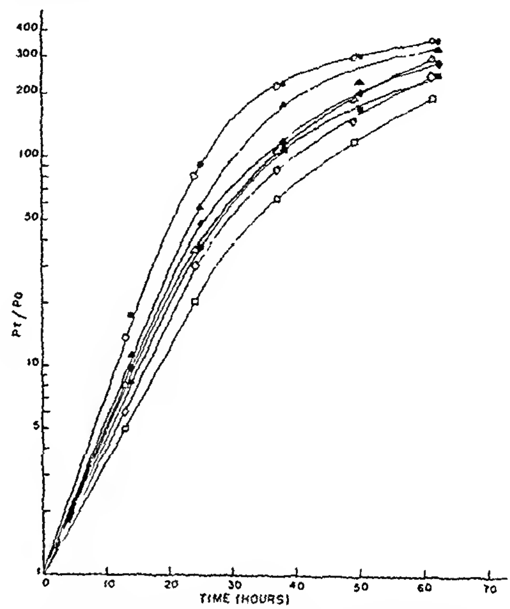

Fig. 5.—The effect of several concentrations of urethane on trained and untrained cells. The time-dependent cell count (P_T) divided by the initial cell count (P_0) is plotted on a logarithmic scale. Normal cells, ○ = control; Δ = 0.07 *M*; ◇ = 0.08, and □ = 0.09 *M*. Trained cells, ● = control; ▲ = 0.07 *M*; ◆ = 0.08 *M*, and ■ = 0.09 *M*.

way, approximately the same number of cells were obtained from each population. The cells were washed three times with 0.005 *M* phosphate buffer (pH 6.9) through the use of an angle head centrifuge. Each centrifugation was carried out for three minutes at 150 × *g*. The washed cells were suspended in 30 ml. of the phosphate buffer to yield a suspension containing about 2.5 mg. of cell protein per ml. (1.7 × 10⁶ normal cells per ml.). After about twenty minutes of time for preparation, the Warburg vessels were loaded with 1.0 ml. of the cell suspension, to which was added 0.5 ml. of the appropriate concentration of urethane in phosphate buffer, additional phosphate buffer was added to give a final volume of 2.3 ml. in the main compartment in all vessels. The center well was charged with alkali (0.2 ml. 20% KOH) and filter paper in the usual manner. After a ten-minute period for temperature equilibrium, the respiration trials were carried out

with air as the gas phase and with shaking at the rate of 130–134 cm. excursions per minute. It was established that the values reported were not limited by the rate of diffusion of oxygen. The uptake of oxygen was measured for a period of either sixty or ninety minutes, during which time the rate was essentially constant except in concentrations in excess of 0.2 *M* wherein the rate fell markedly and the effect of the drug was not reversible.

The rate (µl. O_2/hour/mg. protein) of the endogenous respiration of the normal cell amounted to 33, while that of the trained cell amounted to 34. These almost identical values are in good agreement with values in the literature reported on a dry weight basis (20, 21, 22) when allowance is made for the difference in the manner of expressing the values. Only one set of the many trials carried out is reported in the data presented in Fig. 6. Each point on the sets of curves in Fig. 6 represents the average of the results from two vessels from each of two cultures. Ten concentrations of urethane, ranging from 0.025 *M* to 0 2 *M*, were studied in addition to the non-urethane controls. The results presented in Fig. 6 are in the form of the "mass-action law" plot used by Ormsbee and Fisher: *U* is the rate of oxygen consumption in the presence of a given concentration of urethane; *I* is the difference between *U* and the respiration in the absence of urethane.[1]

As first pointed out by Ormsbee and Fisher, the effect of urethane on the rate of the endogenous respiration is best characterized by two intersecting straight lines. In the case of both classes of cells, the lines intersect at a urethane concentration of about 0.11 *M*. In the results of Ormsbee and Fisher, the break in the curve was observed at 0.1 *M* urethane. The difference in the sensitivity to the drug of the more sensitive respiration, as indicated

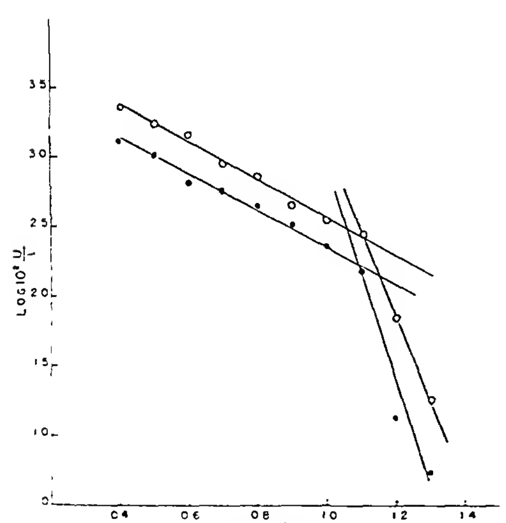

Fig 6.—The effect of urethane on the endogenous respiration of trained and untrained cells. The data are plotted according to the formulation of Ormsbee and Fisher (see text).

[1] According to Fisher (11), $\log (U/I) = \log K - a \log N$, where *K* is the dissociation constant of a drug-enzyme complex, *N* is the concentration of drug, and *a* is the number of molecules of drug uniting with each active enzyme site.

in Fig. 6, is small. At 0.11 *M* urethane the endogenous respiration is depressed 26%, while the respiration of the trained cell is depressed 35%. The differences are less at lower concentrations. At the lowest concentration, the difference amounts to about 4%. In any event, the trained cell is more, rather than less, sensitive to the respiratory depressing effects of urethane. This finding is in contrast to the effects on growth, wherein the trained cell is significantly less sensitive.

Metabolism of Drug.—While these studies had little concern with the mechanism by which the increase in drug resistance took place, it was deemed necessary to exclude the possibility that the improved growth was due to the circumstance that the trained cells metabolized urethane faster and thus caused a reduction in the relative concentration. Due to the greater exposure of the cells to urethane in the growth trials, in contrast to the respiration trials, an increased capacity to metabolize urethane would bias the growth results more than the respiration data. Accordingly, we tested the ability of the trained cell to metabolize urethane.

An amount of urethane to yield a final concentration of 0.08 *M* was added to each of four 750-ml. portions of medium and to each of two 750-ml. volumes of water. Two of the flasks containing medium were inoculated with trained cells and incubated for seventy-two hours, after which the cells were removed by centrifugation. One hundred-milliliter aliquots were then removed from each of the cell-free media and from each of the remaining vessels and extracted repeatedly with ethyl ether by a standardized procedure. The urethane in each of the ether extracts was crystallized with the use of petroleum ether, dried, and weighed. The averages of the duplicate weights from each set of aliquots were: 475 mg. from water; 475 mg. from the plain medium, and 480 mg. from the medium which had supported cell growth. The melting points of the several samples were in the range 48–50°. The agreement in amounts recovered by the standardized procedure indicates that degradation of urethane is not significant and that it is not the mechanism by which training in growth takes place.

DISCUSSION

The results presented in Figs. 1 and 6, pertaining to the control cells, establish the fact that despite some differences in experimental conditions, the strain of *Tetrahymena* used in these experiments responded to urethane similarly to the strain used by Ormsbee and Fisher (6). As in their results, the "mass-action law" plot of the results relating the rates of endogenous respiration to the concentration of urethane is best represented by two intersecting straight lines (Fig. 6). Likewise, the concentration of drug associated with the point of intersection is the same as that which inhibited cell multiplication almost completely. According to them, the respiratory activity suggested by the line with the lesser slope is related in a specific fashion to the process of cell multiplication. The respiratory function represented by the greater slope was considered to be associated with the vegetative activity of the cell. The argument was advanced by us (to present only one possibility) that support for their proposal would be obtained if cells trained to grow in urethane

showed an appropriate decrease in the sensitivity of the respiration to the depressing effect of the drug. The significance of the results would be enhaneed if the change in sensitivity were confined to that portion of the respiration suggested by the lesser slope.

The results presented in Figs. 2, 3, and 5 demonstrate clearly that training in urethane promoted measurable increases in the resistance of the cultures to the growth-inhibiting effect of the drug. However, the results obtained under conditions of poor oxygenation and slow growth (Fig. 2) stand in sharp contrast to the results obtained under conditions of good aeration and more rapid growth. The eell trained in 0.04 *M* urethane under conditions of poor oxygenation developed a complete insensitivity to the growth-inhibiting effect of 0.08 *M* urethane. It is noteworthy that even with the degree of training established relative to the effect of 0.08 *M* urethane, it was not possible to train the organisms to grow, even slowly, in 0.1 *M* urethane. The limited degree of training which we have achieved with *Tetrahymena* is in marked eontrast to the successes achieved by Hinshelwood (13) and co-workers working with bacteria. It is generally recognized that protozoa adapt to the inhibitory effect of most drugs only slowly and not extensively (23).

The results presented in Fig. 6 indicate definitely that neither the more sensitive respiration nor the total endogenous respiration showed a decrease in drug sensitivity as a result of training. Indeed, the adapted cells were slightly more sensitive to the respiratory effect of the drug.

These findings indicate quite definitely that there is no change in respiration, reflected in the endogenous respiration, that would account for the degree of training obtained in the growth studies. In a similar fashion, Preston and Eiler (24) could find no decrease in sensitivity to the respiration depressing effect of pentobarbital in brain slices from rats rendered tolerant to the action of the drug. However, McCashland (25) recently observed an adaptive increase in the respiration, measured under the conditions of growth, in *Tetrahymena* trained to resist the effects of potassium cyanide.

The absence of a rigid coupling between respiration and cell multiplication, however, does not preclude a significant relation between the two processes. An important aspeet of cell respiration, together with the anaerobic metabolism, is the supply of energy it provides for endergonic processes The energy-rich phosphate compounds formed as a result of aerobic oxidations, and to a lesser extent anaerobic oxidations, make possible growth and division of the cell. A coupling between respiration and growth based upon supply and demand for energy-rich compounds is a necessity in the life of aerobic organisms. A significant depression in respiration must be accompanied by a decline in endergonic processes unless compensated by an increase in the rate of the reactions which supply energy under anaerobic conditions. It is possible that just such an increase in anaerobic metabolism may be basic to the improvement in growth observed in our experiments.

The greater resistance to the growth-inhibiting effects of urethane established under the conditions of poor oxygenation and slow growth (Fig. 2) suggests that the principal change in the trained cell is the development of a more significant anaerobic metabolism. Ryley (21) has shown that *Tetrahymena pyriformis* is able to survive and maintain motility under anaerobic conditions The anaerobic metabolism, at least of mammalian cells, is not nearly so sensitive to the inhibitory effects of narcotics as is the aerobic metabolism; indeed, the aerobic glycolysis of brain is increased by narcotics (4). An increase in anaerobic metabolism is a logical consequence of subculturing in relatively anaerobic conditions, especially when the aerobic metabolism has been depressed somewhat by urethane.

The acceptance of an adaptive change in the anaerobic metabolism carries with it the idea that growth and division are inhibited primarily through the action of the drug on respiration. Certainly, if the growth-inhibiting effects are, in part, reversed by the development of an anaerobic metabolism, it is not reasonable that that fraction of the growth could have been inhibited by a direct effect of the drug on the growth process *per se*.

It should be clear that the respiration whose inhibition has been considered to lead to an inhibition of growth is not the respiration which has been measured in these experiments; rather, it is the respiration which is measured under conditions in which cell division is taking place. The possible relation between the two respirations, relative to growth, is not known at this time. Fortunately, such interrelations, together with the behavior of the anaerobic reactions, are susceptible to experimental study.

SUMMARY

1. The effects of urethane on the endogenous respiration and on the rate of cell multiplication has been studied on trained and untrained cells of *Tetrahymena pyriformis* with the view of gaining some understanding of the mode of action of narcotic drugs.

2. The results of the action of the drug on the untrained cells are in good agreement with the earlier findings of Ormsbee and Fisher which suggested a signifieant relation between eell multiplication and respiration.

3. The cells from cultures which had been trained by repeated subeulturing in the drug showed a higher rate of eell multiplication in a given coneentration of drug than did the untrained cells. The differenee was most marked in eultures which have been trained under conditions of poor oxygenation. However, the endogenous respiration of the trained cells in a given coneentration of urethane was no higher than that of the untrained cells.

4. The results are eonsidered in terms of a possible mode of aetion of urethane.

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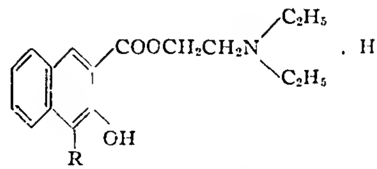
Dialkylaminoethyl Esters of 4-Nitro- and 4-Amino-3-hydroxy-2-naphthoic Acids*

By GEORGE M. SIEGER†, WILLIAM M. ZIEGLER†, DAVID X. KLEIN, and HERMAN SOKOL

The 2-diethylaminoethyl ester of 3-hydroxy-2-naphthoic acid was nitrated in acetic acid solution to yield the corresponding ring-substituted 4-nitro derivative. Catalytic hydrogenation of the nitro compound gave 2-diethylaminoethyl 4-amino-3-hydroxy-2-naphthoate hydrochloride. The anesthetic properties of these nitro and amino derivatives have been studied and compared with unsubstituted 3-hydroxy-2-naphthoic acid esters of the same series.

IN A PREVIOUS PUBLICATION (1) the synthesis of a number of dialkylaminoalkyl esters and amides derived from 3-hydroxy- and 3-alkoxy-2-naphthoic acids and some of their physiological properties were described In continuation of these earlier studies, it was deemed worthwhile to prepare ring-substituted nitro and amino derivatives of the 2 diethylaminoethyl ester of 3-hydroxy 2-naphthoic acid in order to determine whether or not the anesthetic activity could be enhanced with relation to the unsubstituted ester The rationale supporting the possibility for an increased anesthetic effect lies in the fact that the proposed compounds could be considered naphthoic acid analogs of ring-substituted local anesthetics, such as procaine (2-diethylamino-ethyl 4-amino-1-benzoate) and Naphthocaine (2-diethylaminoethyl 4-amino-1-naphthoate) and their related 4 nitro compounds (3, 4). Since it has been demonstrated in several series of the local anesthetics that the introduction of second ring substituents, such as amino or alkoxyl groups, sometimes increases the activity (3–9), there seemed to be a sufficiently good reason for preparing a ring-amino derivative of 2-diethylaminoethyl 3-hydroxy-2-naphthoate for testing as a local anesthetic.

Synthesis of the proposed ring-substituted amino derivative of 2-diethylaminoethyl 3-hydroxy-2-naphthoate was accomplished by nitrating the hydrochloride of this ester (I) in glacial acetic acid under the same conditions described by Gradenwitz (2) for the preparation of methyl 4-nitro-3-hydroxy-2-naphthoate (V) and subsequent reduction of the nitro derivative (II) to the corresponding amino compound (III) by catalytic hydrogenation.



II − R = NO_2
III − R = NH_2

Designation of the nitro and amino groups as ring substituents in the 4-position is consistent with the work of Gradenwitz (2) who obtained the 4-nitro ring derivative of the methyl ester of 3 hydroxy-2-naphthoic acid (V) under conditions similar to those used for nitration of the diethylaminoethyl ester. These structures were established further by hydrolyzing both the nitrated methyl and 2-diethylaminoethyl esters (V and II)

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to give the same acid, namely, 4-nitro-3 hydroxy-2 naphthoic acid (VI) described by Robertson (10)

PHARMACOLOGY

In comparative rabbit corneal anesthetic tests run according to the method of Sollmann (11) and described more fully in our previous publication (1), the 4-nitro and 4-amino ring-substituted derivatives of 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride (II and III) did not appear to be significantly more active than the unsubstituted ester (1) and were less active than the homologous 3-diethylaminopropyl 3-hydroxy-2-naphthoate hydrochloride (Bonacaine-G) (1)

EXPERIMENTAL

2-Diethylaminoethyl 3-Hydroxy-2-naphthoate Hydrochloride (I)—This ester was prepared by the reaction of 3-hydroxy-2-naphthoic acid with the free base of diethylaminoethyl chloride as described in procedure C of our previous publication (1)

2-Diethylaminoethyl 4-Nitro-3-hydroxy-2-naphthoate Hydrochloride (II)—Thirty-two grams (0 1 mole) of 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride (I) was suspended in 350 ml of glacial acetic acid While the suspension was chilled in an ice bath and stirred, 10 ml (*ca* 0 15 mole) of concentrated nitric acid, diluted with 150 ml of glacial acetic acid, was slowly added dropwise so that the temperature remained below 20° After all the nitrating solution had been added the ice bath was removed The temperature of the reaction mixture rose slowly to 29° and its color turned from pale yellow to dark orange. An additional 100 ml of glacial acetic acid was added to the stirred mixture After about twelve hours the mixture was filtered through a sintered-glass funnel to remove the somewhat pasty crude mononitro compound After triturating the crude product with 2B ethanol and heating it under reflux with 250 ml of hot anhydrous ethanol for several hours, the product was filtered The yellow crystalline compound was triturated repeatedly with alcohol-ether, m. p 219-221°; yield, 13 Gm (35 4%)

Anal.—Calcd for $C_{17}H_{21}O_5N_2Cl$: N, 7 6; Cl, 9 6 Found: N, 7 3, Cl, 9 5

2-Diethylaminoethyl 4-Amino-3-hydroxy-2-naphthoate Hydrochloride (III)—Twelve grams (0 0326 mole) of 2-diethylaminoethyl 4-nitro-3-hydroxy-2-naphthoate hydrochloride (II) and 0 3 Gm of platinum oxide catalyst were suspended in 150 ml of ethanol and 10 ml of water The mixture was hydrogenated at room temperature, under an initial pressure of 44 p s i, for six hours. After removal of the catalyst, the mixture was concentrated *in vacuo* at as low a temperature as possible. On chilling the concentrate, gold-yellow needles separated from the solution After filtration, the dried product melted 184 8 to 185 8°; yield, 2.5 Gm. (22 5%). It was soluble in water and much less soluble in small volumes of cold alcohol or acetone

Anal—Calcd for $C_{17}H_{23}O_3N_2Cl$· C, 60 3; H, 6 8, N, 8 3; Cl, 10 5 Found: C, 60 4; H, 7 0; N, 8 4, Cl, 10 3

Methyl 3-Hydroxy-2-naphthoate (IV)—The procedure used for preparing the methyl ester of 3-hydroxy 2-naphthoic acid was essentially a modification of the procedure of Cohen and Dudley (12) The yellow crystalline ester obtained melted 71 to 73° Literature (12) gives 73 to 74°

Methyl 4-Nitro-3-hydroxy-2-naphthoate (V)—The methyl ester of 3-hydroxy-2-naphthoic acid (IV) was nitrated in acetic acid by essentially the same procedure as described by Gradenwitz (2) The mononitro compound was isolated as yellow crystalline needles melting 186 to 189° in a 30% yield The literature (2) gives 188 to 189° as the m p for this compound.

Anal—Calcd for $C_{12}H_9O_5N$: N, 5 68, ester value, 227 mg KOH/Gm Found· N, 5 69, ester value, 234 9 mg KOH/Gm

4-Nitro-3-hydroxy-2-naphthoic Acid (VI)—Twenty grams (0 081 mole) of methyl 4-nitro-3-hydroxy 2-naphthoate (V) was saponified with 400 ml of 10% sodium hydroxide solution diluted with 400 ml of water over a two hour period with the aid of gentle heating until almost a completely red-colored solution was obtained While hot, the solution was filtered The small amount of insoluble material on the filter plate was washed with an additional 200 ml of water The red-colored filtrate was acidified with excess concentrated hydrochloric acid (*ca* 300 ml), causing the color of the filtrate to turn yellow and the precipitation of a yellow-colored product. It was filtered, washed with water, and dried; yield, 16 4 Gm (87%); m p 233 9 to 234 9° Robertson (10) reported 233 to 238° (decompn) as the melting point for this compound

Anal—Calcd for $C_{11}H_7O_5N$· N, 6 02, mol wt, 233 Found: N, 5 74; mol wt, 231 (by potentiometric titration).

Hydrolysis of 2-Diethylaminoethyl 4-Nitro-3-hydroxy-2-naphthoate Hydrochloride (II)—Following the same procedure as described above, 2-diethylaminoethyl 4-nitro-3-hydroxy-2 naphthoate hydrochloride (II) yielded the same corresponding acid (VI), as ascertained by melting point and microanalytical determinations

SUMMARY

1. The 4-nitro and 4-amino ring derivatives of 2-diethylaminoethyl 3-hydroxy-2-naphthoate hydrochloride have been synthesized.

2. Introduction of the 4-nitro and 4-amino substituents into the 3-hydroxy-2-naphthoic acid nucleus of the basic ester did not alter its anesthetic activity significantly.

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In Vivo Fibrinolytic Effect of Various Proteolytic Enzymes: Quantitative Tests Employing Iodine^{131}-Labeled Clots*

By J. L. AMBRUS†, N. BACK†, S. GOLDSTEIN††, C. M. AMBRUS†, and J. W. E. HARRISSON

Purified fibrinogen was labeled with I^{131}. Clots were produced in various blood vessels of dogs with the aid of this material. Radioactivity was continuously registered over the clot. Thus, the clot dissolving activity of potential fibrinolytic agents could be evaluated quantitatively. Four proteolytic enzymes of animal and plant origin were found to be ineffective as fibrinolytic agents.

MANY METHODS have been described to produce experimental thrombosis in animals. In the study of potential fibrinolytic agents, the disappearance of the clot was observed mainly by palpation, visual observation during autopsy, or histologic preparations. All these methods are obviously qualitative. We have developed methods (1, 2) for the quantitative testing of such agents. Fibrinogen was labeled with I^{131} and the radioactivity of the clot produced with this material *in vivo* was continuously recorded over the thrombosed area. Using this method together with conventional procedures, the fibrinolytic activity of trypsin and various preparations of plasmin have been tested. Preparations and dose ranges of plasmin were found which effectively dissolved clots without causing important biochemical changes (3). Nevertheless, because of various difficulties involved in the use of such preparations therapeutically, it was felt that a preliminary screening should be undertaken in search for other effective agents. Four proteolytic enzymes of animal or plant origin were tested in this study: crude pancreatic protease, ficin, papain, and carboxypeptidase.

MATERIALS AND METHODS

Adult mongrel dogs of both sexes were used. They were anesthetized with 25 mg./Kg sodium pentobarbital; this dose was supplemented with further doses as needed. The veins or arteries to be thrombosed were prepared and the segment to be used was isolated between two artery clamps. All side branches were ligated except for one which was cannulated with a polyethylene tube Most of the blood from the isolated segment was withdrawn through this cannula into a syringe containing 0.1 to 0.2 ml. I^{131}-labeled fibrinogen. The mixture was withdrawn and reinjected three times to insure proper mixing. This was followed by the introduction of thrombin in 0.1 ml. saline.

A semi- or completely constricting ligature was placed distal (in arterial clots) or proximal (in venous clots) to the thrombosed area. This served to prevent escape of the clot without being lysed. If such escape nevertheless occurred, the embolic clot particles could easily be detected by scanning the animal with the scintillation counter. Experiments in which this occurred were excluded from evaluation. It is possible that in some studies reported by other investigators in which conventional methods were used, escape of the clot may have been recorded as lysis of the clot. This may explain some positive results in the literature which could not be confirmed by this method.

After about thirty minutes the polyethylene cannula was removed and the side branch ligated; the artery clamps were removed and the vessel was placed in the trough of a specially constructed lead shield which accommodated a scintillation counter. This was connected to a radiation rate meter which, in turn, was connected to a counter as well as to an Esterline recorder. The former registers the total number of counts within any desired time period, while the latter continuously graphs count per minute values. All values in the table refer to radioactive counts per minute.

In preliminary experiments it was shown that decrease in radioactivity registered by these methods is proportional to lysis of the clot. After lysis of the clot with trypsin, which was incorporated into the clot at the time of its formation, radioactivity rapidly decreased in the blood and did not increase to any important degree in any organ. Most of the I^{131} was recovered from the urine. Probably I^{131}-labeled fibrin was broken down to small molecular size peptides which are not taken up by the thyroid gland.

Radioactivity was continuously recorded for at least four hours, and every twenty-four hours thereafter. The dogs received 1 million units penicillin and 250 mg. streptomycin subcutaneously daily, and veterinary Terramycin powder locally. The wound was closed with wound clips and reopened for the daily recordings. In the tables, the values before administration of the agents to be tested and at four, twenty-four, and ninety-six

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We are greatly obliged to Parke, Davis and Co for thrombin and Benadryl, Lederle Laboratories for penicillin, Sharp and Dohme Division of Merck and Co for streptomycin, Chas Pfizer and Co for Terramycin, and Warner-Chilcott Labs for Simplastin

hours were reported If the animal died before the end of one of these periods, the values were recorded in the tables at the nearest of these time periods

As an additional criterion of fibrinolysis, visual observation by transillumination was undertaken The light source of a Metro photoelectric tensiometer was dismounted This provided a powerful light source at the end of a thin, curved, stainless steel tube By holding the light source under the thrombosed segment of the vessel, the presence or absence of fibrin network could be established to some degree The following arbitrary units were used to report these data 3—full clot, no fluid phase, 2—full clot, little fluid phase, 1—decreased clot size, more fluid phase, 0—no clot Figure 1D[1] is a photograph of the experimental setup

Iodine^{131}-labeled fibrinogen was prepared on the basis of a modification of the method of Mihalyi and Laki (4) as described previously (1, 2) Two grams of commercial bovine fibrinogen was dissolved in 100 ml of a 0 1 M phosphate buffer at pH 6 4 To this was added 100 ml cold distilled water This was refrigerated for at least six hours This was then centrifuged and the sediment discarded To the supernatant was added a saturated solution of

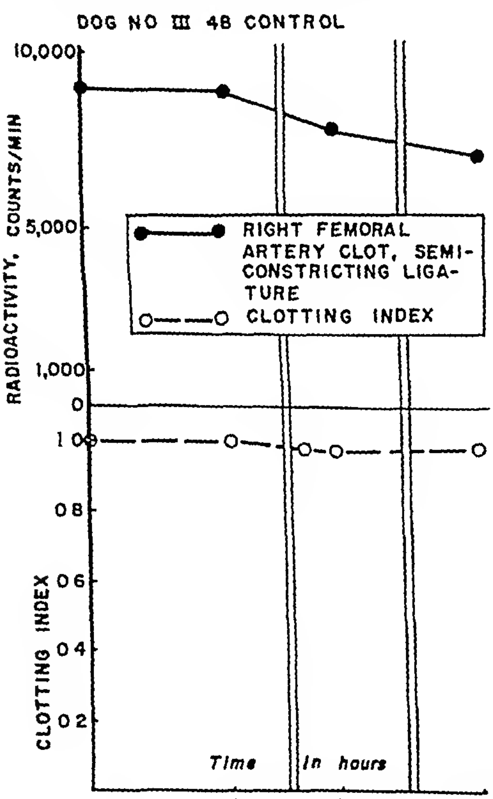

Fig 1—Effect of saline on clotting index and I^{131}-labeled clot in dog

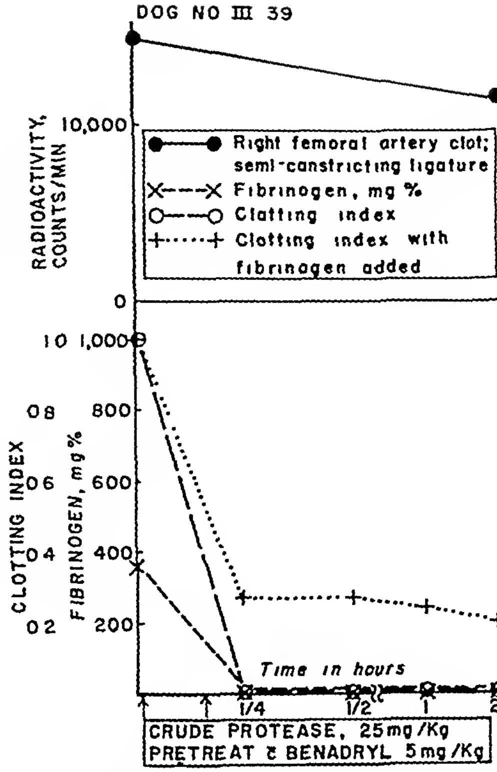

Fig 2—Effect of crude protease, 25 mg /Kg , on clotting index, fibrinogen level, and I^{131} labeled clot in dog pretreated with Benadryl, 5 mg /Kg

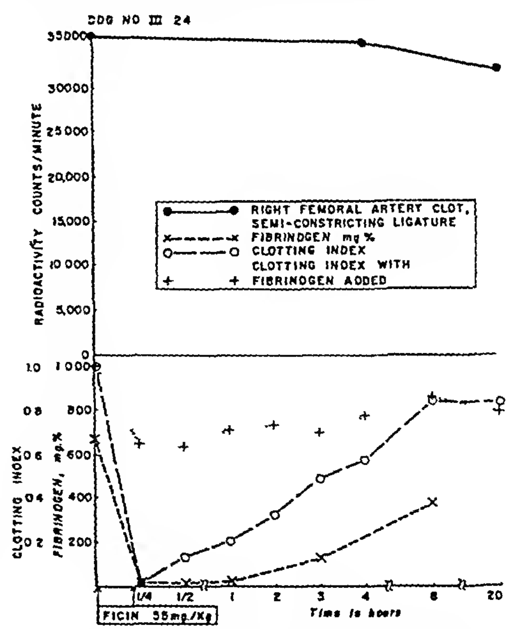

Fig 3—Effect of ficin, 35 mg /Kg , on clotting index, fibrinogen level, and I^{131}-labeled clot in dog

ammonium sulfate equal to $^1/_3$ of the original volume resulting in a final concentration of 25% $(NH_4)_2SO_4$, which caused the precipitation of fibrinogen This was then centrifuged and the precipitate was dis-

[1] Figures and tables identified by ' D ' have been deposited as Document No 5815 with the American Documentation Institute Auxiliary Publications Project Photoduplication Service Library of Congress Washington 25 D C A copy may be secured by citing the Document No 5815 and remitting $1 25 for photoprints or $1 25 for 35 mm microfilm Advance payment is required Make checks or money orders payable to Chief, Photoduplication Service Library of Congress

TABLE I.

Drug and Dose	Time	No of Clots	—Clots Showing a Decrease in Radioactivity of— 0–25%	26–50%	51–75%	76–100%
Control	Up to 4 hr.	25	100 0	0 0	0 0	0 0
	Up to 24 hr	26	88 5	7.7	3 8	0 0
	Up to 96 hr	22	63 5	27 2	9 2	0 0
Carboxypeptidase,	Up to 4 hr	3	66 7	33 3	0 0	0 0
21 7 mg /Kg	Up to 24 hr	3	66 7	33 3	0.0	0 0
	Up to 96 hr	3	0 0	66 7	0 0	33 3
Papain, 35 mg /Kg	Up to 4 hr	6	50 0	50 0	0 0	0 0
Ficin, 35 mg /Kg	Up to 4 hr	6	83 3	16 7	0 0	0 0
Crude protease,	Up to 24 hr	3	66 7	33 3	0 0	0 0
25–35 mg /Kg	Up to 4 hr.	6	66 7	33 3	0 0	0 0

TABLE II

Drug and *in vivo* Dose	*In vitro* Proteolytic Activity Loomis u /mg	—Radioactivity of Clot— Up to 4 hr No of clots	Mean % lysis	Up to 24 hr No of clots	Mean % lysis	Up to 96 hr. No of clots	Mean % lysis
Control saline		25	6 6	26	13 9	22	25 3
Carboxypeptidase, 21 7 mg /Kg single infusion	<0 01	3	15 8	3	19 5	3	30 0
Crude pancreatic protease, single infusion							
25 mg /Kg	53 0	3	23 0				
35 mg /Kg		3	27 3				
Papain, 35 mg /Kg, single infusion,	0 06	6	19 0				
Ficin 35 mg /Kg, single infusion	0 3	6	18 3	3	19 2		

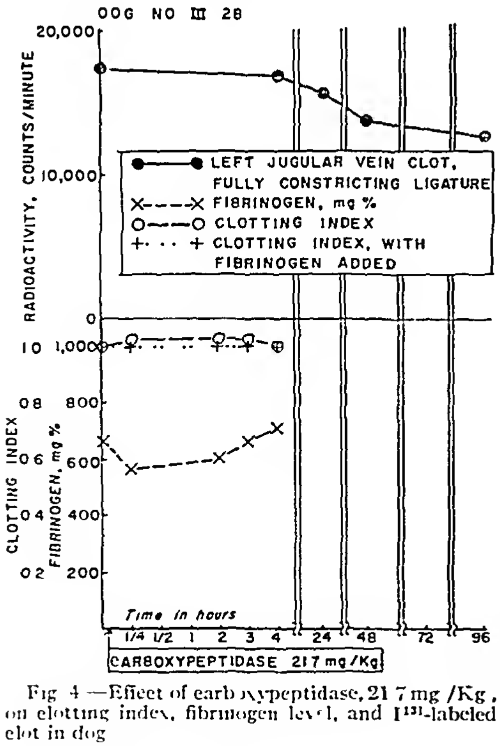

Fig 4 —Effect of carboxypeptidase, 21 7 mg /Kg, on clotting index, fibrinogen level, and I^{131}-labeled clot in dog

solved in 10 ml of distilled water and dialyzed for twenty-four hours against 0 3 M KCl solution in the cold A 0 1 N iodine solution was prepared in a 0 15 M potassium iodide solution To this, 10 mc of I^{131} was added as sodium iodide The solution was incubated at 37° to allow equilibration between the I_2 and the I^{131} ions. The latter solution was usually made up simultaneously with the former one. On the following day, 40 ml. of the fibrinogen solution was mixed in an ice bath with 40 ml borate buffer of pH 8 58 (equal volumes of 0 5 M boric acid and 0 125 M sodium tetraborate) and 100 ml 40% urea solution To this mixture 20 ml of the precooled iodine solution was added After thirty minutes, the remaining free iodine was reduced with a 10% sodium thiosulfate solution Urea, iodide ions, and sodium thiosulfate were then removed by overnight dialysis against double distilled water in the cold Iodinated fibrinogen was then precipitated by 5 ml of 0 5 M acetate buffer (equal volumes of 0 5 M acetic acid and 0 5 M sodium acetate) of pH 4 6 The precipitate was redissolved in 10 ml of distilled water and adjusted to pH 7 5 with 0 1 N sodium hydroxide Fresh I^{131}-labeled fibrinogen was prepared for each experimental series

In all animals, prothrombin times were determined periodically from oxalated plasma with the aid of Simplastin (5) Clotting index was calculated as the ratio of the value before infusion of the compounds under test and the experimental values This same determination was repeated after adding 0 1 ml of a 2 5% fibrinogen solution to the test mixture This procedure was used to get an indication as to the role of decrease in fibrinogen level in changes of the clotting index. Fibrinogen values were determined by the method of Shea (6)

Blood pressure and EKG's were taken in all animals

The following enzymes were tested in this study: pancreatic protease, ficin, carboxypeptidase, and papain Crude lyophilized pancreatic protease was obtained from the Worthington Laboratories Crystalline ficin was supplied by the Mann Research

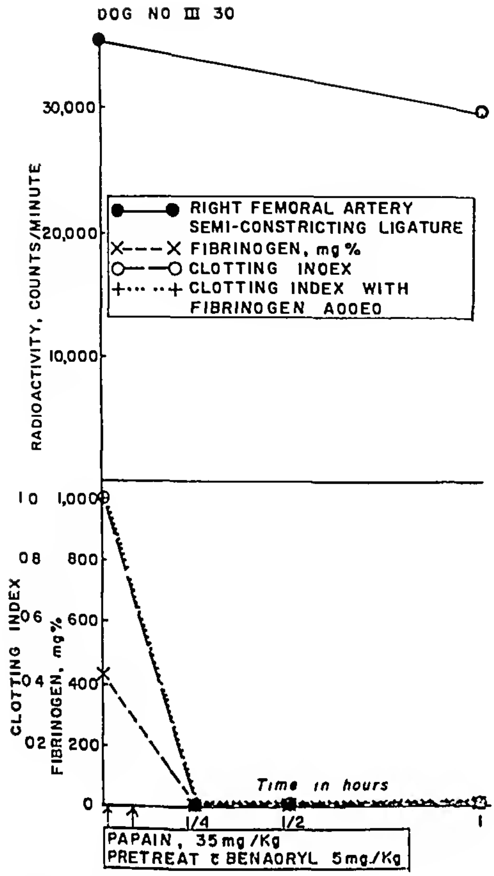

Fig 5—Effect of papain, 35 mg /Kg on clotting index, fibrinogen level, and I^{131} labeled clot in dog pretreated with Benadryl, 5 mg /Kg

Laboratories Carboxypeptidase (triple crystallized) was prepared according to the method of Anson (7) by the Worthington Laboratories Papain was obtained from Merck and Co All of these compounds were tested for *in vitro* fibrinolytic activity by the method of Loomis, George, and Ryder (8) The results of these *in vitro* tests are summarized in Table II The doses given to the animals were chosen to be the maximal quantities which do not cause death within at least four hours The time of infusion was selected to be the shortest period which does not cause irreversible fall in blood pressure

RESULTS

Figure 1 shows the results of a control experiment It can be seen that only slight spontaneous fibrinolysis occurs in ninety six hours Figure 2 shows the results of an experiment in which 25 mg /Kg of pancreatic protease has been administered There is a sharp fall of fibrinogen level and clotting index, even in the presence of added fibrinogen Although in many experiments the blood became incoagulable, no significant fibrinolysis occurred Table ID[1] indicates the abbreviations used in all tables Table IID[1] presents the data of the experiments with pancreatic protease and the corresponding control studies Figure 3 shows the results of an experiment with ficin Again, there can be seen a sharp fall in clotting index and fibrinogen values, but no evidence is present of fibrinolysis Table IVD[1] contains the detailed data of the ficin experiments together with controls Figure 4 graphs the results of an experiment with carboxypeptidase No significant change is observed in clotting index or fibrinogen level, nor is there more fibrinolysis than in the control animals The detailed data are tabulated in Table VD[1] This same table contains results obtained with papain A typical papain experiment is shown in Fig 5 Again, there is a precipitous fall in fibrinogen level and clotting index with and without fibrinogen but there is no evidence for fibrinolysis

Table I groups all the above results according to the percentage of clots exhibiting the indicated percentage lysis values at various time intervals Table II summarizes all experiments by indicating the mean percentage lysis at various time intervals obtained with the compounds tested This table also contains the results of *in vitro* fibrinolytic assays It can be seen that none of the enzymes tested had any important *in vivo* fibrinolytic activity The slight increase over control values shows relation to the *in vitro* activity

Except for carboxypeptidase, all of the compounds tested produced fall in blood pressure This could not be prevented by pretreatment with 5 mg /Kg Benadryl The EKG changes were mostly insignificant occasionally T wave reversion was seen with protease

DISCUSSION AND SUMMARY

Four proteolytic enzymes, crude pancreatic protease, ficin, carboxypeptidase, and papain, have been tested for *in vivo* fibrinolytic activity upon intravenous administration in dogs A quantitative method was used for evaluation based on the production of clots with I^{131} labeled fibrinogen and continuous recording of radioactivity over the thrombosed blood vessel segment None of the enzymes tested produced significant lysis All except carboxypeptidase decreased clotting index and fibrinogen level This often resulted in complete incoagulability of the blood.

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A Note on the Volatile Oil from the Fruits of *Lomatium Grayi**

By FRANK A. PETTINATO†, LOUIS FISCHER, and NATHAN A. HALL

MANY SPECIES of the genus *Lomatium* grow prolifically in some areas of the state of Washington and have been the subject of only a limited number of studies. A literature review revealed that *Lomatium Grayi* was one species which had not been previously investigated. As part of a study of this genus in an effort to find constituents of medicinal or economic value, a preliminary investigation of the volatile oil from the fruits of *Lomatium Grayi* Coult. and Rose was undertaken.

The fruits of the plant were collected in June 1956 near Klickitat, Washington, dried in air, and ground in a Wiley mill through a 1-mm. screen immediately before use. Two hundred-thirty milliliters of oil was steam distilled from 7.1 Kg. of the ground fruit for a yield of 3% on an air-dried basis. The pale yellow oil was dried over anhydrous sodium sulfate and the physical constants and the results of the chemical analysis shown in Table I were obtained.

TABLE I.—PHYSICAL AND CHEMICAL PROPERTIES OF *L. Grayi* OIL

Specific gravity, 25°/25°	0.8782
Optical rotation, 25°	−16.05
Refractive index, 23°	1 4848
Acid number	0.83
Ester number	24 5
Ester number (after acetylation)	46 9
Esters (as bornyl acetate)	8.6%
Alcohols (as borneol)	13.4%
Aldehydes and ketones (by hydroxylamine method, assuming average M. W. of 150)	4 0%

TABLE II.—PHYSICAL PROPERTIES OF *L. Grayi* OIL FRACTIONS

Fraction	Boiling Range, °C.	Distillate, %	$(n)^{20}_{D}$	d^{25}_{25}	$[\alpha]^{25}_{D}$
1	To 46	1	1.4659	...	−19.30
2	46–53	1	1.4742	...	−10.60
3	53–55	2	1.4822	0.848	− 7.00
4	55–57	24	1.4822	0 852	− 4.90
5	57–58	24	1.4820	0.849	− 4.00
6	58	16	1.4805	0.848	− 3.10
7	58–60	11	1.4782	0.848	− 1.80
8	60–75	1	1.4772	...	− 2.80
Residue and loss	. .	20	...	...	...

Seventy-five milliliters of the oil was fractionally distilled from a Todd Fractionation Assembly, Model A equipped with a 0.5 x 90 cm. column (with monel spiral) at 10 mm. pressure and a 10:1 reflux ratio. The fractions and their physical characteristics are shown in Table II.

The further characterization and identification of the constituents of the oil are in progress.

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Supported in part by the Fund for Medical and Biological Research of the state of Washington
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The authors wish to thank Dr A E Kruckeberg, Dept. of Botany, University of Washington, Seattle, and Dr. Lincoln Constance, Dept of Botany, University of California, Berkeley, for the botanical identification.

Communication to the Editor

Gastric Antacids

Sir:

Subsequent to the publication of the article, "The Antisecretory and Antipeptic Activity of Gastric Antacids in the Histamine-Stimulated Rabbit" by Packman, *et al.* [THIS JOURNAL, 48, 46(1959)], we noticed several errors which should be corrected. They are as follows:

$$\text{VPN Index} = \frac{\begin{array}{c}\%\ \text{Reduction}\\ \text{in gastric}\\ \text{secretion}\end{array} \times \left[1 + \begin{array}{l}\%\ \text{Reduction}\\ \text{in proteo-}\\ \text{lytic}\\ \text{activity}\end{array} + \begin{array}{l}\%\ \text{Animals}\\ \text{maintained}\\ \text{in pH range}\\ \text{3–5.5}\end{array}\right]}{\text{Acid consuming power}}$$

Immediately following the VPN indexes should be: DASC, 8.9; calcium carbonate, 4.0; sodium bicarbonate, 4.1; and aluminum hydroxide, 0.1.

We regret that these errors were not corrected in galley proof. We wish to assure you that these errors resulted from our oversight and not yours or that of the proofreader.

E. W. PACKMAN,
D. D. ABBOTT,
B. TRABIN,
and J. W. E. HARRISSON
La Wall and Harrisson Research Laboratories,
Philadelphia, Pa.

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Sabadilla Alkaloids VII*

Sabatine and Its Alkamine Sabine

By HYMAN MITCHNER and LLOYD M. PARKS†

A corrected molecular formula is proposed for the monoacetate alkaloid sabatine, $C_{29}H_{47-49}O_8N$, and its alkamine sabine, $C_{27}H_{45-47}O_7N$. It has been shown that sabine neither isomerizes nor possesses the masked α-ketol system present in other highly oxygenated alkamines isolated from Sabadilla or from other *Veratrum* species.

THE ALKALOID SABATINE was isolated in 1951 by Hennig, Higuchi, and Parks (1) as a constituent of *Schoenocaulen officinale*, more commonly called Sabadilla. Sabatine was reported to be a monoacetate ester of the alkamine sabine and was obtained with chloroform of crystallization present. Sabine was obtained in the crystalline form as a sulfate salt. Because of the nature of the crystalline materials it was not possible to report unequivocal analytical data and this complicated the determination of empirical formulas and further characterization of the two substances. Molecular formulas approximating $C_{31}H_{53}O_8N$ for sabatine and $C_{29}H_{51}O_7$ for sabine were postulated.

In the present study of sabatine and sabine crystallized from solvents other than those used previously, new data have indicated that sabine is probably a C_{27} alkamine ($C_{27}H_{45-47}O_7N$). The C_{27} carbon skeleton appears to be common to the alkamines isolated from *Veratrum* species (2). All of the other alkaloids previously isolated from Sabadilla, veratridine, cevadine, cevacine, and vanilloylveracevine were esters of the alkamine veracevine, $C_{27}H_{43}O_8N$. Veracevine in common with the other highly hydroxylated alkamines: germine, protoverine, and zygadenine possesses a masked α-ketol system capable of undergoing isomerization (2, 3). However, hydrolysis experiments and countercurrent distributions indicate that sabine is unique in the highly oxygenated alkamines in that it appears to be a stable alkamine, incapable of undergoing isomerization and therefore probably lacking a masked α-ketol system.

EXPERIMENTAL

Sabatine from Acetone-Water.—A 1-Gm. sample of sabatine, crystallized from chloroform, was dissolved in a small volume of acetone. Water was added until the solution became cloudy. Upon warming and adding a few drops of acetone the solution cleared, and after being allowed to stand overnight 700 mg. of crystalline sabatine was obtained which did not give a chlorine test upon fusion with sodium. The sabatine was dried at 100° under vacuum for sixteen hours, m. p. 256–258°.

	$C_{29}H_{47}O_8N \cdot {}^1/_2H_2O$ Calcd.	$C_{29}H_{49}O_8N \cdot {}^1/_2H_2O$ Calcd.	Found
C	63.71	63.48	63.45, 63.81
H	8.85	9.19	9.19, 9.06

Sabatine from Ethyl Acetate.—Five hundred milligrams of sabatine, crystallized from acetone-water, was dissolved in 50 ml. of ethyl acetate. The solution was evaporated to 10 ml. and placed in a refrigerator. After two days, 410 mg. of crystalline sabatine was obtained, m. p. 256–258°.

	$C_{29}H_{47}O_8N$ Calcd.	$C_{29}H_{49}O_8N$ Calcd.	Found
C	64.78	64.52	64.42, 64.79
H	8.82	9.15	8.75, 8.84

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Volatile Acid Determination on Sabatine.—This was carried out on 30-mg. samples of the pure alkaloid by the procedure of Niedrl and Niedrl (4), and in two determinations it showed 0.96 and 0.98 moles of a volatile acid. As a check upon the complete hydrolysis of the alkaloid, the procedure of Nash and Brooker (5) was also used and showed only 1 mole of a volatile acid.

Investigation of the Acids of Sabatine.—A sample of sabatine (27.8 mg.) was hydrolyzed with 1 ml. of *p*-toluenesulfonic acid as in the volatile acid determination. The hydrolysate was washed into a 50-ml. beaker with 1 ml. of water, thoroughly triturated with 2 Gm. of silicic acid, and slurried with chloroform. The slurry was added to the top of a previously prepared partition column (internal phase 20 ml. of water plus 20 Gm. of silicic acid; chloroform as the external phase). The column was developed using a series of solvents increasing in polarity as suggested by Marvel and Rands (6). Fractions of 8 ml. were collected and, after the addition of 1 ml. of water and a drop of *m*-cresol purple indicator, titrated with 0.00976 N sodium hydroxide. Fraction 21–32 showed an acid content corresponding to 0.93 moles of acid. No other acidic material was found in the 72 fractions collected.

The acidic fractions, 21–32, were combined and the chloroform and aqueous phases were separated. The chloroform was washed with 5 ml. of 0.01 N sodium hydroxide and the washings added to the combined aqueous phases. *p*-Toluenesulfonic acid was added to the aqueous solution until it became acid to litmus. The solution was then distilled and the first 30 ml. of the distillate was collected. A micro Duclaux determination was performed on the distillate. Four-milliliter fractions were collected and titrated with 0.00976 N sodium hydroxide. The Duclaux values obtained were 6.48, 7.07, 7.37, corresponding to acetic acid.

Hydrolysis of Sabatine and the Isolation of Sabine

Hydrolysis with Sodium Ethoxide.—To a solution of 200 mg. of sodium in 30 ml. of absolute ethanol 200 mg. of sabatine was added. The solution was heated under reflux for four hours. After cooling, the hydrolysis mixture was adjusted to a pH of 6.5 with tenth normal hydrochloric acid and the ethanol distilled *in vacuo*. The concentrate was made strongly alkaline with 5 N potassium hydroxide and extracted with three 25-ml. portions of chloroform. The chloroform was dried over anhydrous sodium and evaporated to dryness *in vacuo* on a steam bath. The residue was taken up in 30 ml. of hot ether, filtered, and evaporated to about 5 ml. On standing, 135 mg. of large cubic crystals formed which were contaminated with a brown color. The crystalline material was difficultly soluble in ether, and for recrystallization it was necessary to dissolve it in a small volume of chloroform and evaporate under reduced pressure to dryness on a steam bath before dissolving the material in hot ether. The crystals frothed at 105–110° and melted at 173–176°. The infrared spectrum indicated that an ester band was not present (Fig. 1). The molecular weight studies (Table II) indicated that solvent of crystallization must have been incorporated.

	$C_{27}H_{45}O_7N \cdot C_4H_{10}O$ Calcd.	$C_{27}H_{47}O_7N \cdot C_4H_{10}O$ Calcd.	Found
C	65.49	65.11	65.09, 65.11
H	9.77	10.04	9.62, 9.81

Upon drying at 117° under reduced pressure, the sabine from ether lost its shiny appearance. The

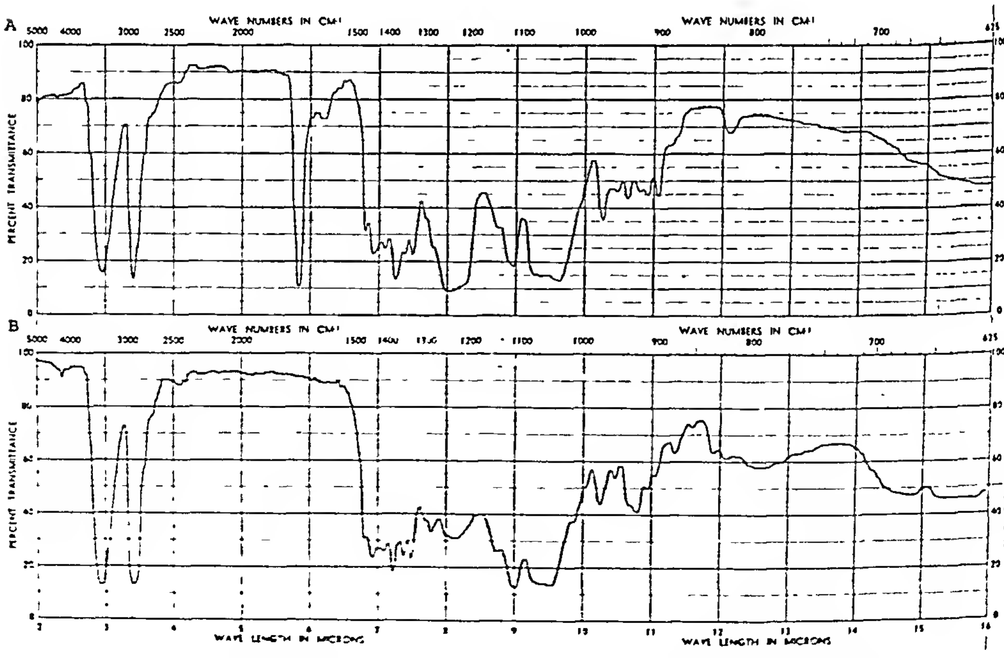

Fig. 1.—Infrared spectra of: (A) sabatine in chloroform solution and (B) sabine in chloroform solution.

infrared spectrum of this material was almost identical with the sabine from ether that was dried at 100°. However the sabine dried at this higher temperature no longer foamed at 105–110°, but still melted at 173–176°. The molecular weight (Table II) was considerably lower after drying at 117°.

	$C_{27}H_{45}O_7N$ Calcd.	$C_{27}H_{47}O_7N$ Calcd.	Found
C	65.42	65.16	65.09, 65.24
H	9.16	9.52	8.85, 9.10

Mild Alkaline Hydrolysis.—An 870-mg. sample of sabatine was dissolved in 25 ml. of methyl alcohol and 5 ml. of 1 *N* sodium hydroxide. The solution was refrigerated at 0° for twenty-four hours and then distilled under reduced pressure to about 5 ml. The concentrate was extracted with ten 5-ml. portions of chloroform. After combining the chloroform extracts, the chloroform was washed with 5 ml. of water, dried over anhydrous sodium sulfate, and evaporated under reduced pressure to dryness on a steam bath. The residue was taken up in 40 ml. of hot ether and filtered. On cooling, 577 mg. of crystalline material was obtained which was identical in all respects to the sabine isolated from the sodium ethoxide hydrolysis.

Methanolysis of Sabatine.—A 300-mg. sample of sabatine was treated in a similar manner to the procedure used for the methanolysis of cevacine (7). The sabatine was dissolved in 10 ml. of methanol and 5 ml. of water, and allowed to stand at room temperature for twenty hours. The methyl alcohol and water were distilled *in vacuo*. The residue was dissolved in 40 ml. of chloroform which was boiled to dryness to remove any traces of water. All attempts to crystallize the white residue were unsuccessful. The infrared spectrum of the material showed the presence of an ester band but other peaks were not distinct.

Countercurrent Investigation of Sabatine Hydrolysis Mixtures

From Sodium Ethoxide Hydrolysis.—The reaction mixture analyzed was the ether mother liquor remaining after removal of the crystalline sabine in the sodium ethoxide hydrolysis as described above. The ethereal solution was evaporated to dryness and the residue was dissolved in 10 ml. of chloroform. This chloroform solution was distributed for 65 transfers in a Craig[1] countercurrent apparatus. The lower phase was chloroform and the upper phase was 0.5 *M*, pH 8.1 phosphate buffer, with 10 ml. of each phase per tube. The alkaloid was analyzed by a nonaqueous titration procedure (8). Figure 2 shows the separation obtained. The tubes containing the peaks were collected and combined in two fractions. The aqueous phases were made very strongly alkaline with 5 *N* potassium hydroxide and thoroughly shaken. The chloroform phases were then withdrawn, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The amount of alkaloidal material from each peak was insufficient for crystallization but their infrared spectra were identical to sabatine and sabine for the first and second peaks, respectively.

A second sodium ethoxide hydrolysis was carried out on 200 mg. of sabatine. However, after extracting the total alkaloidal components in chloroform, the chloroform solution was evaporated to 10 ml. and distributed for 57 transfers in the Craig countercurrent apparatus. Chloroform was again used as the lower phase and 0.5 *M*, pH 8.15 phosphate buffer was used as the upper phase. Tubes 39–57 were combined and 25 ml. of 5 *N* potassium hydroxide was added. After shaking and removing the chloroform, the aqueous layer was washed with a 200-ml. portion of chloroform. The combined chloroform solutions were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residue was dissolved in 40 ml. of hot ether and evaporated to about 10 ml. On standing, large cubic crystals formed which were identical to sabine in all respects. Sabatine was present in the first peak but only in a very small amount.

From Methanolysis.—A 200-mg. sample of the methanolysis reaction mixture isolated as described above was dissolved in 10 ml. of chloroform and distributed in a Craig countercurrent apparatus for 100 transfers. Chloroform was used as the lower phase and 0.5 *M*, pH 8.1 phosphate buffer as the upper phase. The distribution obtained is shown in Fig. 3. Tubes 0–30 and 70–100 were combined in two separate fractions. To each fraction 50 ml. of 5 *N* potassium hydroxide was added and the phases shaken. The chloroform phases were removed and the aqueous phases were each shaken with two 100-ml. portions of chloroform. The chloroform washings were combined with their respective original chloroform extracts, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The alkaloidal material from tubes 70–100 was taken up in hot ether, filtered, and evaporated to about 5 ml. On standing, cubic crystals were obtained for which analytical data agreed in all respects with sabine. The alkaloidal material from tubes 0–30 was taken up in a very small volume of chloroform and seeded with sabatine previously isolated from chloroform solution. On standing, needle crystals were obtained which were identical in all respects to sabatine.

Reaction of Sabine, Sabatine, Cevadine, Cevacine, Veracevine and Its Isomers With Triphenyltetrazolium Chloride.—Five milligrams each of sabatine, sabine, veracevine, cevagenine, cevine, cevacine, and cevadine was placed in separate test tubes, and to each was added 1 ml. of a 1% aqueous triphenyltetrazolium chloride solution and 1 ml. of 0.1 *N* sodium hydroxide. A control was prepared with the two solvents alone. The solutions were allowed to stand at room temperature and were observed at various time intervals to note any color changes. After seventy-two hours the solutions of cevine, sabatine, sabine, and the control were sealed in ampuls and heated at 100°, again noting any color changes. The results are expressed in Table I.

Periodic Acid Titration of Sabatine and Sabine.[2]—Samples of sabatine (19.0 mg.) and sabine (20.8 mg.) were dissolved in a solution of 10 ml. of 0.05 *M* periodic acid and 5 ml. of 5% acetic acid, and then

[1] The Craig apparatus used was a 200 plate, robot driven instrument, manufactured by H. O. Post, Scientific Instrument Co., Maspeth, N. Y.

[2] This titration was kindly performed by Dr. M. Neeman of the Chemistry Department, University of Wisconsin.

TABLE I.—REACTION OF TRIPHENYLTETRAZOLIUM CHLORIDE WITH CEVADINE, CEVACINE, VERACEVINE CEVAGENINE, CEVINE, SABATINE, AND SABINE

	Reaction at 25°							
	5 min	30 min	2.5 hr	6 hr.	12 hr	24 hr.	48 hr.	72 hr.
Cevadine			+	+	+	++	+++	+++
Cevacine		+	++	++	+++	+++	+++	+++
Veracevine	+	++	+++	+++	+++	+++	+++	+++
Cevagenine	+++	+++	+++	+++	+++	+++	+++	+++
Cevine				+	+	+	+	+
Sabatine							.	...
Sabine							...	. .
Control						..	...	...

Reaction at 100° (A Continuation of the Above Reaction But in Sealed Ampuls)

	30 min	6 hr	12 hr	21 hr	48 hr	72 hr.
Cevine	+++	+++	+++	+++	+++	+++
Sabatine				Yellow	Yellow	Yellow
Sabine				Yellow	Yellow	Yellow
Control				Yellow	Yellow	Yellow

\+ Very faint pink color ++ Definite pink color +++ Red color

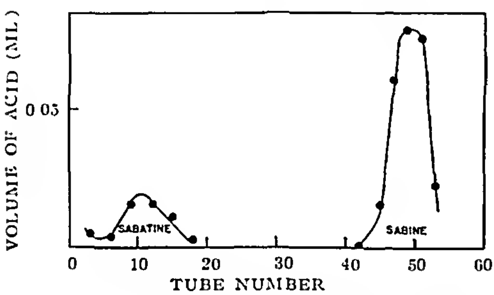

Fig. 2.—Countercurrent distribution of the alkaloidal constituents in the mother liquor from the sodium ethoxide hydrolysis of sabatine after the removal of crystalline sabine—chloroform *vs.* pH 8.1 phosphate buffer.

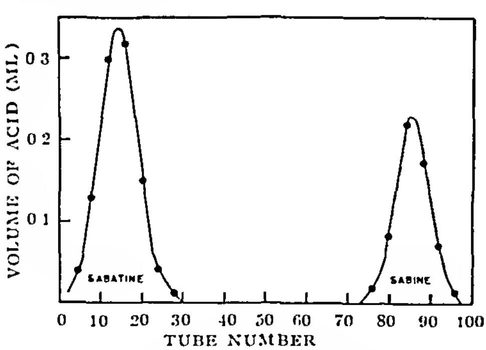

Fig. 3.—Countercurrent distribution of the products from the methanolysis of sabatine—chloroform *vs.* pH 8.1 phosphate buffer.

diluted to 25 ml. At time intervals of one hour and fifteen minutes, two hours and thirty minutes, and twenty-six hours, 5-ml. aliquots of the reaction mixture were withdrawn, treated with 5 ml. of 0.06 *N* sodium arsenite, 5 ml. of a saturated aqueous solution of sodium bicarbonate, and titrated with 0.02 *N* iodine solution. The periodic acid consumed by the sabine corresponded to 1.9 moles, the acid consumed by the sabatine to 0.9 moles at all time intervals.

Equivalent Weight Titrations of Alkamines and Alkaloids.—Samples of alkaloidal substances of from 4 to 10 mg. were dissolved in 4 ml. of reagent grade chloroform. To this solution, three drops of thymol blue indicator (0.1% solution in butyl alcohol) were added and the solution titrated with 0.01122 *N* perchloric acid in dioxane to the permanent appearance of a pink color, the color changing from yellow to pink. The perchloric acid was standardized by a similar titration in chloroform against reagent diphenylguanadine. The results are summarized in Table II.

DISCUSSION

The chloroform of crystallization in sabatine crystallized from chloroform can be removed by recrystallization from acetone-water. However, elemental analyses and molecular weight determinations indicated that, even after drying at 100° the sabatine from acetone-water contained water of crystallization. Upon recrystallization from ethyl acetate the water was removed and solvent-free sabatine which melted at 256–258° was obtained, and for which the formula $C_{29}H_{47-49}O_8N$ was in agreement with elemental analyses and molecular weight determinations. A volatile acid determination on sabatine and chromatography of the hydrolytic products indicated that sabatine was indeed a mono acetate ester as it had already been suggested (1).

Hydrolysis of sabatine with hot sodium ethoxide or with cold methanol and sodium hydroxide yielded an alkamine fraction which could be crystallized from ethyl ether to give a material which frothed at 105–110° and melted at 173–176°. The infrared spectrum of this material showed no trace of an ester band (Fig. 1). In almost all respects it appeared to be identical to the alkamine sabine that Hennig, Higuchi, and Parks had obtained previously in an amorphous form from ether (1). The molecular weight of the sabine crystallized from ether was greater than the molecular weight of the parent ester sabatine (Table II). It appeared that the alkamine had taken on solvent of crystallization. If the sabine was dried under reduced pressure at a temperature of 117°, the alkamine lost its shiny appearance and gave a material which did not froth at 105–110° but did melt at 173–176°. The infrared spectrum for the sabine before drying at 117° was almost identical to the infrared spectrum

TABLE II.—EQUIVALENT WEIGHT TITRATIONS OF ALKAMINES AND ALKALOIDS

Material	Formula	Wt. of Sample, mg.	Equivalent Wt. Calcd.	Equivalent Wt. Found
Veracevine	$C_{27}H_{43}O_8N$	6.05, 4.94	510	515, 516
Cevine	$C_{27}H_{43}O_8N$	7.92, 9.07	510	509, 517
Germine	$C_{27}H_{43}O_8N$	5.11, 6.96	510	508, 505
Sabatine (from ethyl acetate)	$C_{29}H_{47-49}O_8N.{}^1/_2H_2O$	6.34, 7.86	538–540	533, 536
Sabine (from ether)	$C_{27}H_{45-47}O_7N \cdot C_4H_{10}O$	8.00, 5.40	570–572	558, 562
Sabine (after drying at 117°)	$C_{27}H_{45-47}O_7N$	6.41, 6.10	496–498	496, 500

after drying. The molecular weight of the sabine after drying corresponded to that which would have been expected for sabine based on the loss of an acetate group from the proposed sabatine formula (Table II). The elemental analyses and molecular weight determinations were in good agreement for the formula $C_{27}H_{45-47}O_7N$.

It has been shown that many of the alkamines such as germine, veracevine, protoverine, and zygadenine (2, 3) undergo an alkali induced isomerization. There existed the possibility that sabine, with a very similar molecular formula to the above alkamines, might undergo similar isomerization. Following the crystallization of sabine from the sodium ethoxide hydrolysis of sabatine, the alkaloidal content of the mother liquor was recovered and investigated with a 65-transfer countercurrent distribution as shown in Fig. 2. Only two peaks were obtained and these corresponded to unhydrolyzed sabatine and to sabine. Methanolysis of sabatine gave a mixture that could be resolved by countercurrent distribution only into two peaks from which sabatine and sabine could be isolated (Fig. 3). No indication of any isomeric alkamines was obtained under either the strong or the mild hydrolytic conditions.

Auterhoff and Kraft (9) have shown that the masked hemiketol systems which occur in veracevine and germine can be reduced with triphenyltetrazolium chloride to produce a red reaction product. Under conditions such that cevadine, the angelic acid ester of veracevine, and the isomeric alkamines, veracevine, cevagenine, and cevine, all reacted with the triphenyltetrazolium chloride, sabatine and sabine failed to reduce the reagent. This failure and the presence of only a single hydrolysis product under both strong and mild hydrolytic conditions suggested not only that sabine is the parent and only alkamine of the ester sabatine but also that the masked hemiketol system found with veracevine and germine (3) is absent in sabine.

Periodic acid titration of sabine showed a take-up corresponding to 2 moles of the acid, whereas sabatine consumed only 1 mole. The acetate group in sabatine could be assumed to be blocking one of the adjacent hydroxyl pairs.

The tendency of sabatine and sabine to pick up solvent of crystallization placed a great amount of stress on the analytical data that could be obtained and particularly on molecular weight determinations. With the small samples of alkaloidal material used in molecular weight studies, nonaqueous potentiometric titrations did not give consistent results. However, a nonaqueous titration procedure similar to the one used in the analysis of countercurrent distributions (8) was found to give results with less than a 2% error. This was checked with pure alkamine samples of known constitution. All molecular weight determinations have been summarized in Table II.

SUMMARY AND CONCLUSIONS

1. It has been confirmed that sabatine, an ester alkaloid isolated from Sabadilla, is a monoacetate ester. New analytical data on the crystalline alkaloid suggested corrected molecular formulas of $C_{29}H_{47-49}O_8N$ for sabatine and $C_{27}H_{45-47}O_7N$ for the alkamine sabine.

2. Under both mild and strong hydrolytic conditions, sabatine yielded only the one alkamine, sabine. Both sabatine and sabine failed to reduce triphenyltetrazolium chloride reagent. The failure of the alkamine to isomerize or to reduce the TTC reagent indicated the absence of a masked α-ketol structure that has been found in the other highly oxygenated alkamines isolated from Sabadilla and other *Veratrum* species.

3. There exists two adjacent hydroxyl pairs in sabine that are susceptible to periodate oxidation and only one pair in sabatine, indicating that the acetate ester group is blocking one of these hydroxyl pairs.

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A Radiometric Method for Determination of Absorption of Ammoniated Mercury from Ointments*

By DONALD L. SORBY and ELMER M. PLEIN

Ammoniated mercury-Hg^{203} was prepared from mercuric chloride-Hg^{203}. The labeled ammoniated mercury was incorporated into ointment bases and used to determine absorption of ammoniated mercury through intact and abraded skin of rats. Twenty-four hours after application of the ointment, both kidneys were removed and assayed for mercury content by beta and gamma radiometric procedures. The two radiometric methods were found to give comparable results.

THE ANALYTICAL DETERMINATION of a medicinal substance in tissues of laboratory animals following absorption from an ointment base is often difficult due to the small amounts of medicinal substance encountered and to the presence of interfering biological material.

Radioisotopes have been used by several investigators to study drug absorption through the skin. The following are representative works of this type of study: Cyr, *et al.* (1), Skauen, *et al.* (2), and Plein and Plein (3) used sodium radioiodide-I^{131} to determine absorption of sodium iodide from various ointment bases. Neesby, *et al.* (4), used sulfur-S^{35} to determine absorption from polythionate solutions applied to the skin of laboratory animals. Wernsdorfer (5) determined the amount of absorption of mercuric chloride-Hg^{203} through intact skin of guinea pigs.

In many methods, gamma-emitting isotopes have been used in determining the amount of medicinal substance in tissue. Often it is desired to study an isotope which emits beta radiation only. Self-absorption of beta particles by the tissue thus becomes a problem which is not experienced in counting gamma radiation. Digestion of tissue samples is then necessary, but is often tedious and time-consuming.

In this experiment it was desired to introduce a simple and rapid method for determining the amount of beta-emitting substance in tissue. Mercury-Hg^{203} was selected for two reasons. Because it emits both beta and gamma radiation, results obtained by beta methods could be conveniently verified by gamma counting of samples. Work of other investigators (6–10) has shown that the usual colorimetric procedures for determining mercury[1] in tissue are difficult and not of the desired selectivity (3) in the concentration ranges encountered. It was expected that the radiometric method would be more favorable than usual methods for studying absorption of mercurials and would give more nearly correct results.

EXPERIMENTAL

The method of Leblond, *et al.* (11), for the assay of beta-emitting isotopes in tissues was adapted for determination of the percutaneous absorption of mercury-Hg^{203} from ointments.

Preparation of Labeled Ammoniated Mercury.—Mercury-Hg^{203} was obtained as a solution of mercuric chloride containing in each ml. 17.5 mg. of mercuric chloride and 1.5 mc. of radioactivity. For preparation of ammoniated mercury-Hg^{203}, 1.5875 Gm. mercuric chloride (reagent grade) was dissolved in 25 ml. of warm water and cooled. Mercuric chloride-Hg^{203} solution (6.8 ml.) was added and the resulting solution poured, with stirring, into 5.2 ml. of cold 10% ammonium hydroxide. The precipitate was filtered from solution, washed with 40 ml. of 0.5% ammonium hydroxide solution, and dried in a desiccator. The isotope yield was nearly 100%.

Preparation of Standard Solution of Ammoniated Mercury-Hg^{203}.—A solution was prepared by dissolving 49.5 mg. of ammoniated mercury-Hg^{203} in 1.5 ml. of concentrated hydrochloric acid and adding distilled water to make a volume of 10 ml. One ml. of the solution made to a volume of 100 ml. with distilled water produced a dilution which contained 39.4 μg mercury per ml. This diluted solution was used as the standard for all mercury determinations.

Preparation of Ointments and Application to Laboratory Animals.—Three ointments were prepared for application to laboratory animals. Each ointment contained ammoniated mercury-Hg^{203} (5%) and liquid petrolatum (3%). The base for ointment A was white ointment, U. S. P. XV; for ointment B, hydrophilic petrolatum, U. S. P. XV; and for ointment C, the base contained stearyl alcohol (3%), white wax (8%), wool fat (15%), and white petrolatum (74%). Ointments were also prepared for use on control animals. For these ointments an equivalent amount of ointment base was substituted for the ammoniated mercury-Hg^{203}.

These ointments were applied to rats following the procedure of Plein and Plein (3). Twenty-four hours after application of the ointment the kidneys were removed for determination of mercury content.

According to Lang, *et al.* (7), mercury accumulates predominantly in the kidneys following percutaneous absorption. It was recommended that

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[1] The term "mercury" is used to describe all chemical combinations of mercury in tissues. All calculations were based on the quantity of atomic mercury rather than its salts.

the quantity of mercury appearing in the kidneys twenty-four hours after application of the ointments be used as an index of the relative amounts of absorption from different ointments.

Preliminary Preparation of Samples for Counting.—The kidneys from each rat were ground together to a fine state of suspension using an A. H. Thomas teflon pestle tissue grinder. The suspensions were made to a 10-ml. volume with distilled water and refrigerated until used for assay. The suspensions produced in this manner were fine enough to flow from a serological pipet.

Determination of Mercury Content of Samples from Gamma Activity.—The kidney samples were prepared for gamma counting by pipetting 1 ml. of tissue suspension into a 1-dram screw-cap vial. The activity of the samples was counted with a well type scintillation counter.[2] The mercury content of the sample was determined by comparing its activity with the activity of a known quantity of standard ammoniated mercury-Hg^{203} solution.

Determination of Mercury Content of Samples from Beta Activity.—One ml. of the kidney tissue suspension was pipetted into each of two fired stainless steel cup type, Tracer Lab E-20 1 inch by $^5/_{16}$ inch, planchets. One-tenth milliliter of standard ammoniated mercury-Hg^{203} solution was added to one planchet and 0.2 ml. of 1% sodium sulfide ($Na_2S \cdot 9H_2O$) solution was added to both planchets. The preparations were mixed by blowing air through a pipet onto the surface and were set aside to dry. To determine the activity of a known amount of mercury-Hg^{203}, 0.1 ml. of standard ammoniated mercury-Hg^{203} solution was pipetted into each of three fired planchets and 0.2 ml. of the 1% sodium sulfide solution was added to each planchet. When all samples appeared dry, they were dried for an additional twelve to twenty-four hours in a desiccator. The activity of the samples was then determined,[3] corrected for self absorbance and background, and compared with the activity of the standard solution. Following is a sample calculation of the mercury content of a kidney tissue suspension.

Suspension + added standard (standard = 1,500 c. p. m.)	= 3,000 c. p. m.[4]
Suspension	= 2,000 c. p. m.
Activity of added standard in the sample	= 1,000 c. p. m.

Activity of suspension corrected for self absorbance: $(1{,}000/1{,}500) = (2{,}000/X)$; X = 3,000 c. p. m.

Amount of mercury in the total sample: $3.94 \times (3{,}000/1{,}500) \times 10 = 78.8\ \mu g$.

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[2] The gamma activity of the samples was counted at 1,150 v. in a Tracer Lab P-20 scintillation detector with a 1-in. NaI(Tl) crystal connected to a Tracer Lab Autoscaler.

The authors wish to thank the Anatomy Department of the University of Washington for use of scintillation counting equipment.

In this research all samples were counted for a sufficient number of counts to have probable error in counting of 2% or less. Whenever necessary, corrections were made for decay loss. Duplicate determinations were carried out on all samples. The determination was repeated if there was nonagreement between duplicates of the same sample.

[3] The beta activity of the samples was determined at 1,400 v. with a Tracer Lab TGC-2 end window (1.6 mg./cm.^{2}) Geiger-Müller counter connected to a Tracer Lab SC-51 scaler equipped with a Tracer Lab SC-42 preset timer.

[4] Counts per minute referred to as c.p.m.

Absorption Studies on Rats with Ammoniated Mercury-Hg^{203}.—White, female Sprague-Dawley rats weighing from 180.5 to 220 Gm. were treated as described above. Ointments A, B, and C were used for intact skin studies and an abraded skin study was also carried out using ointment A. Each medicated ointment was applied to a group of six rats and in each series a control ointment was applied to a seventh rat.

The mercury content of the kidneys from each rat was determined by gamma counting and by beta counting. The results of these studies are summarized in Table I.

TABLE I.—RESULTS OF ABSORPTION STUDIES ON RATS WITH AMMONIATED MERCURY-HG^{203}

Ointment	Mean Absorption in μg — Beta Determination	Mean Absorption in μg — Gamma Determination
A (Intact skin)	8.6 ± 2.0[a]	8.7 ± 2.1
A (Abraded skin)	39.0 ± 12.0	41.3 ± 12.4
B	9.2 ± 2.9	9.4 ± 3.1
C	10.5 ± 1.9	10.8 ± 2.3

[a] Standard deviation of the mean of six kidney samples was calculated by the formula $s = \sqrt{(1/n - 1)\Sigma d^2}$

DISCUSSION

Beta counting of tissue samples was complicated by the volatility of mercurial compounds. Addition of 0.2 ml. of 1% sodium sulfide ($Na_2S \cdot 9H_2O$) solution to the planchets was found to be the most satisfactory method for reducing the volatility.

An attempt was made to determine the amount of penetration of mercury into the skin from the area of application of the ointment. The procedure used for the beta determination of mercury in the kidney samples was found to be unsatisfactory for use with the skin samples. It was thought that nonuniform sampling caused failure in agreement of the gamma and beta determinations of the skin samples. This nonagreement was probably due to inability to reduce the skin to a fine suspension using the teflon pestle tissue grinder. Heating the skin samples with a few drops of 90% hydrogen peroxide produced more uniform suspensions. After destroying the excess peroxide with 3% potassium permanganate, the radiometric assay was carried out and gave more favorable results. It is probably necessary to adjust the method of producing a uniform sample to the particular tissue being studied. Once a uniform sample is obtained, the general method for determination of the amount of isotope should be applicable.

A rather large variation was occasionally encountered between the amounts of absorption for different rats receiving the same type of ointment. These variations were also reported by other authors (3, 7–9).

Statistical analysis of the results was carried out using the t-test for determination of significant differences between sample means. The level of significance was chosen as $P = 0.10$. The results obtained by beta counting were compared with results of gamma counting of the same kidney samples. No significant differences were found between the two methods for the intact skin studies on ointment A, ointment B, and ointment C ($P > 0.50$). The

results of beta counting of mercury absorbed from ointment A through abraded skin of rats differed significantly from results obtained by gamma counting ($0.05 > P > 0.01$)

CONCLUSIONS

The radiometric method for determining absorption of ammoniated mercury through intact and abraded skin is simple and is accurate for determining small amounts of mercury encountered under the conditions of this type experiment. The procedure is suitable for testing dermatologic vehicles for their effect on absorption of drugs. The general method for beta determination is probably applicable to any mercurial used in ointment form and, with certain modifications, could be adapted to isotopes of other medicinals commonly studied for their release from ointment bases

Since the gamma-counting method is uncomplicated by volatility and self-absorbance and since it is relatively easy to carry out, it is preferred to the beta-determination method The beta technique requires less expensive equipment and would be advantageous in cases when isotopes emit only beta radiation.

The radiometric procedure is more simple than colorimetric methods and requires less sample treatment in carrying out the determination of mercury. The results confirm that the method of Leblond, *et al.* (11), is advantageous in studies of percutaneous absorption of medicinals and the theory that a radioactive tracer technique for determining absorption of ammoniated mercury is practical for use.

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Interaction of Preservatives With Macromolecules II*

Correlation of Binding Data With Required Preservative Concentrations of *p*-Hydroxybenzoates in the Presence of Tween 80

By F. D. PISANO and H. B. KOSTENBAUDER

Data are presented to show that the preservative activity of *p*-hydroxybenzoic acid esters in the presence of Tween 80 is primarily a function of the concentration of unbound preservative. Prediction of required preservative concentrations from a knowledge of the degree of interaction of the preservative with nonionic surfactants is illustrated.

A RATHER HIGH degree of association between *p*-hydroxybenzoic acid esters and Tween 80 has previously been demonstrated (1), and several investigators (2, 3) have suggested that such an interaction might account for the observed interference of nonionic surfactants with the preservative acitivity of the parabens and other phenolic preservatives Although there is ample evidence that such inhibition does occur (2–10), the majority of these studies have not been designed in a manner which would permit a direct correlation of preservative activity and the degree of binding of the preservative by the surfactant.

Aoki, Kamata, Yoshioka, and Matsuzaki (9) presented data which indicated a relationship between solubility of the parabens in Tween 20[1] solutions and antifungal activity in these solutions. Aoki, Kamata, Matsuzaki, and Nakatani (11) conducted further studies on this system, using very dilute solutions of Tween 20 (<0 1%) In these dilute solutions there is little binding of the preservative, and the decrease in surface tension produced by the Tween may become a relatively important factor in determination of the required preservative concentration.

In the present investigation minimum inhibitory concentrations of methylparaben were obtained for *Aerobacter aerogenes* and *Aspergillus niger* in control media and in media containing several concentrations of Tween 80[2] The

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[1] Tween 20 is polyoxyethylene sorbitan monolaurate Atlas Powder Co Wilmington, Del
[2] Tween 80 is polyoxyethylene sorbitan monooleate Atlas Powder Co Wilmington, Del

increased quantity of paraben required in the presence of Tween 80 was compared with the concentration which would be predicted from a knowledge of the degree of binding of the methylparaben by the Tween, and the assumption that the preservative activity is a function of the concentration of the unbound or free paraben.

A simple, chemically defined culture medium was chosen for these studies to avoid, so far as possible, any competing interactions of the preservative with components in the medium. Special precautions were taken with *Aspergillus niger* because, as noted in a report by Barr and Tice (12), this organism is capable of hydrolyzing the ester linkage in Tween, with liberation of the free fatty acid. In order to investigate any possible direct influence such as this, the minimum inhibitory concentration for *Aspergillus niger* was observed when the organism was in contact with the Tween and this hydrolysis was observed to proceed, and also when the organism was separated from the Tween by a nylon membrane and the hydrolysis was thus prevented.

EXPERIMENTAL

Reagents.—Recrystallized methyl *p*-hydroxybenzoate,[3] m p. 129–131°; Tween 80, a commercial sample; all other chemicals were reagent grade.

Microorganisms.—The organisms selected for these studies were *Aerobacter aerogenes*[4] and *Aspergillus niger*[5] It was necessary that the organisms chosen should be capable of growth in synthetic media and have an optimum growth temperature of about 30°, since binding data were readily available at this temperature.

Aerobacter aerogenes, in addition to satisfying the previous conditions, has the further advantage that growth can readily be followed either by titration of acid produced or by turbidity measurements.

Selection of the Culture Medium.—Evidence indicating binding of phenols by amides (13) and nylon (1) suggests the possibility that when studies are carried out using common media containing substances such as peptone or beef extract, some of the drug being studied may be bound to the protein or amino acids in the media To avoid such interactions the following medium was selected:

Dextrose, anhydrous	5 00%
NaCl	0 50%
$MgSO_4 \cdot 7H_2O$	0 02%
$K_2HPO_4 \cdot 3H_2O$	0 10%
$NH_4H_2PO_4$	0 10%
Distilled water to make	100%
pH of medium 6 5	

The possibility of an interaction between methylparaben and dextrose was investigated by determining the solubility of methylparaben in dextrose solutions of varying concentration. It was found that there was no increase in methylparaben solubility in solutions containing up to 10% dextrose, and it was thus concluded that there was not a significant interaction between dextrose and the preservative.

Sterilization.—It was found that sterilization could be effected without caramelization of the dextrose if the salt solution was autoclaved separately and then added aseptically to the solution of dextrose, Tween, and paraben previously sterilized by autoclaving at 15 pounds for twenty minutes. It was also found that caramelization did not occur if the solutions were combined and then autoclaved at 15 pounds for fifteen minutes, followed by rapid cooling.

The possibility of hydrolysis of methylparaben upon autoclaving was investigated by determining the ultraviolet absorbance of methylparaben in both water and medium before and after autoclaving. No change was observed in the ultraviolet absorption spectrum Solutions of Tween 80 were titrated with sodium hydroxide solution before and after autoclaving with no increase in the amount of sodium hydroxide required for neutralization to a phenolphthalein end point It was thus assumed that the preservative and the Tween were stable to autoclaving.

Influence of Culture Medium on Interaction of Paraben with Tween.—To determine any influence which the culture medium might have on the binding of the preservative by the Tween 80, comparative dialysis studies were carried out in water and in the culture medium according to the procedure described in an earlier publication (1).

Studies on Aerobacter Aerogenes.—Growth studies were carried out in Pyrex test tubes, 25 x 150 mm. Tubes, each containing 20 ml. of medium with the desired concentration of Tween and paraben, were inoculated with a loopful of a twenty-four-hour culture of the bacteria grown in the synthetic medium. These tubes were then maintained at a temperature of 30°. Growth was followed by determination of turbidity at varying time intervals, using a Klett-Summerson photoelectric colorimeter with a No 42 blue filter. Observations were made on two tubes at each time interval.

In addition, tubes containing medium with 0, 2, 4, and 6% Tween 80 and a series of concentrations differing by 0 005% in the plain medium and 0.02% in the Tween-containing medium, were prepared in duplicate and stored at 30° for daily gross visual observation over a period of one month. Inoculations of control and Tween-containing samples were made at the same time to avoid any variation in the inoculum.

Studies on Aspergillus niger.—The procedure for the inoculation of tubes with *Aspergillus niger* was identical to that for *Aerobacter aerogenes* with the exception that the inoculum consisted of a loopful of a spore suspension from a one-week culture of *Aspergillus niger* in the synthetic medium. Growth was observed visually as positive or negative in duplicate samples of the medium containing 0 and 7% Tween 80 and a series of paraben concentrations differing by 0 005% in the plain medium, and 0 02% in the Tween-containing medium.

Additional studies were carried out in which

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[3] Methyl Parasept, purified, Heyden Newport Chemical Corp., New York
[4] ATCC No 8308
[5] ATCC No 10254.

the organism was separated from the Tween by a nylon membrane Tween, paraben, and 20 ml of medium were placed inside nylon sacks (1) which permitted free passage of the paraben, but which were impermeable to the Tween, the microorganism, and probably to any esterase produced by the micro organism These bags were then placed in ground glass stoppered bottles containing 20 ml of medium, and the entire assembly was sterilized by intermittent sterilization to avoid damage to the nylon membrane The bottles were then agitated for fifteen hours at a temperature of 30° This agitation permitted equilibrium to be established between the paraben and the macromolecule The liquid outside the bag was then inoculated and the bottles were maintained at 30° for observation of growth

RESULTS AND DISCUSSION

If the failure of normal inhibitory concentrations of the parabens in the presence of Tween 80 can be attributed primarily to the association between preservative and surfactant, and if the inhibition of microbial growth in absence of Tween 80 can be obtained by a paraben solution of a thermodynamic activity corresponding to less than saturation, it would follow that in the presence of any concentration of Tween 80 there must be some concentration of paraben less than saturation which will prevent microbial growth

In any aqueous system containing Tween 80 and methylparaben, the total paraben present will consist of paraben bound to Tween and the free or unbound paraben

$$\text{Paraben} + \text{Tween} \rightleftharpoons \text{Paraben Tween}$$

It has been shown previously that for any given Tween 80 concentration the ratio of total to free methylparaben is a constant, r (1) If the preservative activity of the paraben can be assumed to be due to the unbound or available form, then it should be possible to calculate required preservative concentrations for any Tween 80 solution by multiplying the required concentration of free paraben by the appropriate r value For these calculations it was assumed that the minimum inhibitory concentration of paraben determined in the absence of Tween corresponds to the concentration of free paraben required Thus, for any given concentration of Tween (Total paraben)/(Free paraben) × Required free paraben = Required preservative concentration

Influence of Medium on Binding—It was found that the interaction of the methylparaben with Tween 80 was not identical in a medium of distilled water and in the culture medium employed in these studies As shown in Fig 1, the paraben interacts with the Tween to a higher degree in the presence of the medium The results for the binding in distilled water are in good agreement with those previously reported It is therefore necessary to take into account the nature of the culture medium, even when it is a very simple synthetic medium, if the binding data are to be correlated with microbiological results

Inhibitory Concentration for Aerobacter Aerogenes—Inhibitory concentrations were obtained

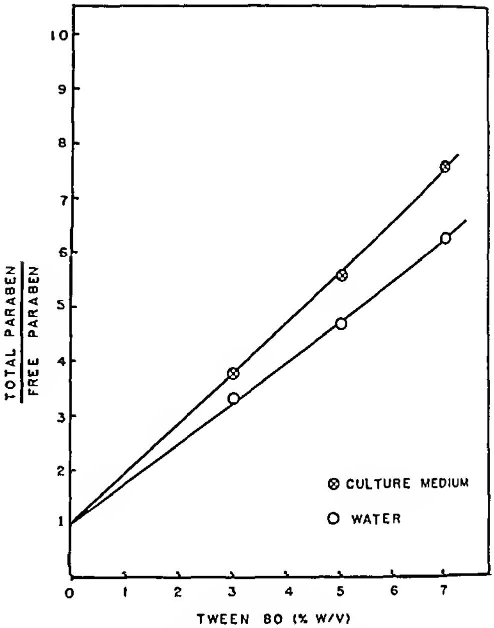

Fig 1—Binding of methylparaben by Tween 80 in water and in the culture medium, 30°

for *Aerobacter aerogenes* in the plain medium and in media containing various concentrations of Tween 80 Higher concentrations were required in the presence of Tween 80, and these experimental concentrations were compared with the values which were predicted from a knowledge of the degree of interaction between paraben and Tween

Figure 2 illustrates the influence of varying concentrations of methylparaben on the growth of *Aerobacter aerogenes* in a medium containing 5% Tween 80 The final inhibitory concentrations in these studies were determined by taking as the inhibitory concentration the range between the lowest paraben concentration not showing growth after one month, and the next lower paraben concentration

Table I shows the minimum inhibitory concentration of methylparaben as a function of Tween 80 concentration

TABLE I—INHIBITORY CONCENTRATION OF METHYLPARABEN IN THE PRESENCE OF TWEEN 80, OBSERVED FOR ONE MONTH

Organism	Tween 80 %	Methylparaben %
Aerobacter aerogenes	0	0.075–0.080
Aerobacter aerogenes	2	0.18–0.20
Aerobacter aerogenes	1	0.28–0.30
Aerobacter aerogenes	6	0.10–0.12
Aspergillus niger	0	0.045–0.050
Aspergillus niger	7	0.32–0.31

Inhibitory Concentration for Aspergillus niger. The inhibitory concentration of methylparaben for *Aspergillus niger* in the plain medium was determined as 0.045–0.050% The inhibitory concentration in the presence of 7% Tween 80 was 0.32–0.34%, when the organism was in contact with the Tween and when the organism was separated from

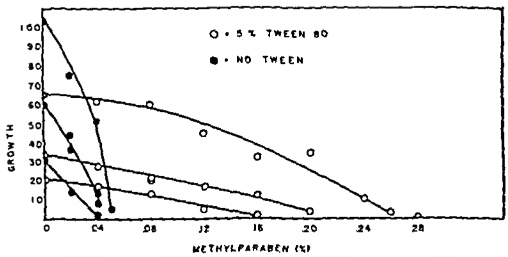

Fig. 2.—A typical growth curve for *Aerobacter aerogenes* in the presence of varying concentrations of methylparaben. The curves show increasing growth after one, two, and seven days, respectively, in plain medium and in medium containing 5% Tween 80. Growth is represented on an arbitrary scale based on turbidity measurements.

the Tween by a semipermeable membrane, but in contact with the paraben, the inhibitory concentration was found to be in the range 0.326–0.346%. The paraben bound by the nylon was taken into account in these calculations. The results suggest that the principal effect of the Tween is to bind the preservative, and any direct influence of Tween on the growth of the organism seems to be negligible so far as required preservative concentration is concerned.

It should be noted that in the studies utilizing the nylon membrane the absence of Tween outside the nylon membrane in concentrations detectable by analytical methods was confirmed, but it was not possible to prevent contamination by minute traces of the surfactant and the resulting decrease in surface tension in the external solution.

Correlation of Binding Data and Inhibitory Concentration.—Figure 3 shows a comparison of the experimental inhibitory concentrations of methylparaben in Tween 80 and the concentrations predicted from a knowledge of the inhibitory concentration in plain media and the ratio of total to free methylparaben at the given Tween concentration. The solid line in Fig. 3 represents a slope of 1, and would indicate agreement between experimental and predicted inhibitory concentrations. The predicted concentrations obtained by multiplying the required concentration in plain media by the r factor were slightly in excess of the experimental values. However, as illustrated in Fig. 4, the concentration of free paraben required to inhibit *Aerobacter aerogenes* in any Tween concentration studied was a constant, approximately 0.065%. This value is slightly less than the concentration of 0.075–0.080% required in plain media, and it is possible that this decrease in required concentration of free paraben may be due to the surface tension depression in Tween 80 solutions. Aoki, Kamata, Matsuzaki, and Nakatani (11) found that in extremely dilute solutions of Tween 20, where binding is probably negligible, lower concentrations of preservative were required than was the case in the absence of Tween. This effect would account for the deviation of the experimental values and predicted values illustrated in Fig. 3.

Although there is a dearth of data concerning the ratio of total to free preservative in the presence of nonionic surfactants, such information can often be obtained from solubility data (1). Thus the data of Chakravarty, Lach, and Blaug (14) for the solubility of methyl and propyl *p*-hydroxybenzoates

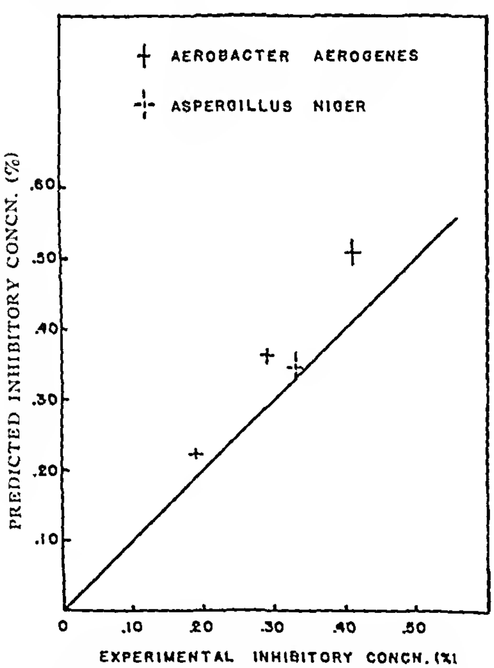

Fig. 3.—A comparison of predicted and experimental inhibitory concentrations of methylparaben in the presence of Tween 80.

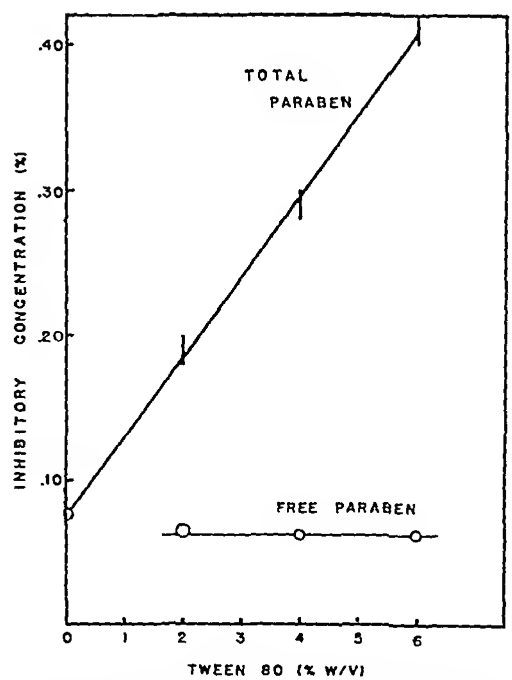

Fig. 4.—A comparison of the total methylparaben concentration and the free methylparaben concentration required to inhibit growth of *Aerobacter aerogenes* in the presence of Tween 80.

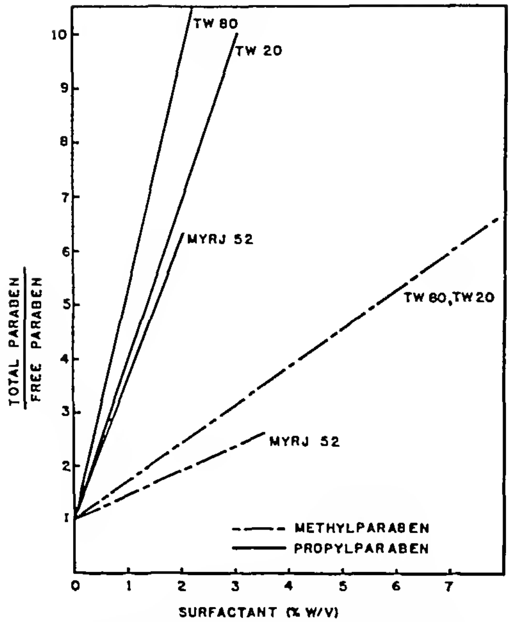

Fig 5—Binding of methylparaben and propylparaben by several representative surfactants of the polyoxyethylene type Data for Tween 80 are from Patel and Kostenbauder (1), data for Myrj 52 were calculated from the solubility study of Chakravarty, Lach, and Blaug (14), and data for Tween 20 were calculated from antifungal concentrations reported by Aoki, *et al* (9)

in Myrj 52[6] can be employed to calculate the ratio of total to free preservative as a function of the concentration of Myrj 52 These data are illustrated in Fig 5 for comparison with the binding exhibited by Tween 80

The ratios of total to free paraben in the presence of Tween 20, shown in Fig 5, were estimated from inhibitory concentrations reported by Aoki, Kamata, Yoshioka, and Matsuzaki (9) These determinations were made in modified Sabouraud media, but the results seem to indicate that the binding of the parabens by Tween 20 would be quite similar to that of Tween 80 and Myrj 52

It is significant that the interaction of propylparaben and other relatively hydrophobic esters with Tween is much greater than the interaction of the methyl ester Thus, for a given concentration of surfactant, a much greater proportion of the total propylparaben will be in an inactive form than would be the case with the methyl ester Aoki, *et al* (9), noted that while the higher esters such as propyl and butyl were effective in lower concentration than the methyl ester in water, in the presence of surfactants this relationship is sometimes reversed It should be possible to select the most suitable preservative for a given system, utilizing data such as those presented in Fig 5

[6] Myrj 52 is polyoxyl 40 stearate Atlas Powder Co Wilmington Del

SUMMARY

1 The preservative activity of *p* hydroxybenzoic acid esters in the presence of nonionic surfactants is primarily a function of the concentration of unbound preservative

2 The *p* hydroxybenzoates can be employed as efficient preservatives in the presence of nonionic surfactants, but the proper concentrations must be employed This study suggests that these concentrations can be estimated by multiplying the usual preservative concentration by the ratio of total to free paraben at any given surfactant concentration

3 Hydrophobic esters such as propyl and butyl are usually effective in lower concentrations than the methyl ester when employed in aqueous systems, but in the presence of surfactants of the polyoxyethylene type this relationship might sometimes be reversed

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Interaction of Preservatives With Macromolecules III*

Parahydroxybenzoic Acid Esters in the Presence of Some Hydrophilic Polymers

By GRACE M. MIYAWAKI†, N. K. PATEL‡, and H. B. KOSTENBAUDER

The magnitude of any intermolecular association occurring in aqueous solution between methyl- and propylparaben and several hydrophilic polymers was determined. Parabens were found to interact to some extent with polyethylene glycol 4000, methylcellulose, polyvinylpyrrolidone, and gelatin. There was no evidence for a significant degree of interaction between the parabens and carboxymethylcellulose or tragacanth. The significance of these results relative to preservation of solutions containing these polymers is considered.

PREVIOUS STUDIES in this series (1, 2) have been concerned with the rather significant interaction of preservatives with nonionic surfactants of the polyoxyethylene type and the importance of considering such interactions when determining proper preservative concentrations. Several articles published in recent years indicate that some thought has been given to interactions of preservatives with macromolecules such as tragacanth (3,4), methylcellulose (3, 5), carboxymethylcellulose (3), Irish moss (3), and polyethylene glycols (6). The purpose of the present investigation was to determine the extent of any such interactions which might occur between p-hydroxybenzoic acid esters and some representative macromolecules, and thus obtain an evaluation of the relative importance of such binding in determining required preservative concentrations.

Studies were performed on polyethylene glycol 4000, methylcellulose, polyvinylpyrrolidone, gelatin, carboxymethylcellulose, and tragacanth, using solubility and equilibrium dialysis techniques to detect any possible association between the parabens and the macromolecules.

EXPERIMENTAL

Reagents.—Recrystallized methyl p-hydroxybenzoate,[1] m. p. 128–128.5°; recrystallized propyl p-hydroxybenzoate,[2] m. p. 96–98°; polyethylene glycol 4000;[3] methylcellulose 15 c. p. s.;[4] carboxymethylcellulose, low viscosity;[5] tragacanth;[6] gelatin, derived from an acid-treated precursor;[7] and polyvinylpyrrolidone.[8] The concentrations of all macromolecular dispersions are expressed on a w/v basis and have been corrected for moisture content as determined by drying a sample of the material to constant weight.

Equilibrium Dialysis Method.—The equilibrium dialysis technique employed in the present study is essentially the same in principle as that used by Klotz, Walker, and Pivan (7) and Karush and Sonenberg (8) in investigating the binding action of proteins, and by Higuchi and co-workers (9, 10, 11) in their studies of the interaction between some macromolecules and pharmaceuticals. The experimental technique was identical to that used by Patel and Kostenbauder (1) in studying the interaction of parabens with Tween 80,[9] with the exception that the membrane employed in these studies was cellulose dialyzer tubing.[10]

The dialysis method was employed for the study of methylcellulose, polyvinylpyrrolidone, gelatin, carboxymethylcellulose, and tragacanth.[11] Twenty-five ml. of the polymer solution under study was placed in each cellophane bag. Each bag was then tightly tied and placed in a wide-mouth glass-stoppered bottle containing 25, 50, or 75 ml. of an aqueous paraben solution. The capped bottles were then agitated for fifteen hours at constant temperature. For the more viscous tragacanth dispersions a longer period of equilibration was required and the above method was modified in that the paraben was dissolved in the tragacanth dispersion and the bottles containing these samples were agitated for several days until constant assay results were obtained for the external solution. The method of assay and the treatment of data have been described previously (1).

The volume of the solution outside the membrane was measured, after equilibration, to permit correction of the polymer concentration in the event a volume change had occurred. Important volume changes were noted only in the carboxymethylcellulose solutions.

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[1] Methyl Parasept, purified, supplied through the courtesy of Heyden Newport Chemical Corp., New York.

[2] Propyl Parasept, purified, supplied through the courtesy of Heyden Newport Chemical Corp., New York.

[3] "Carbowax" 4000, supplied through the courtesy of Union Carbide Chemicals Co., New York.

[4] Methocel 15 c. p. s., supplied through the courtesy of Dow Chemical Co., Midland, Mich.

[5] CMC, 70 premium low, supplied through the courtesy of Hercules Powder Co., Wilmington, Del.

[6] S. B. Penick & Co., New York.

[7] Pharmagel A, Pharmagel Corp., New York.

[8] Plasdone, supplied through the courtesy of Antara Chemicals, New York.

[9] Tween 80 is polyoxyethylene (20) sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

[10] A. H. Thomas, Philadelphia, Pa., average pore diameter 48 Ångström.

[11] Tragacanth contains a small fraction of dialyzable material; but any error introduced by neglecting this fraction is probably insignificant, particularly in view of the nature of the data obtained.

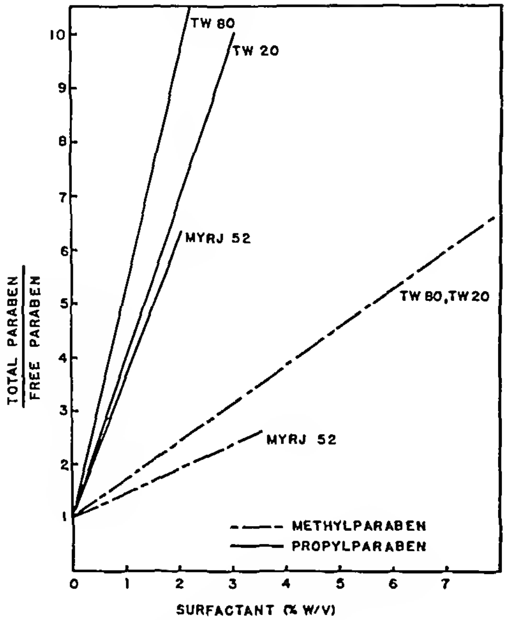

Fig 5—Binding of methylparaben and propylparaben by several representative surfactants of the polyoxyethylene type Data for Tween 80 are from Patel and Kostenbauder (1), data for Myrj 52 were calculated from the solubility study of Chakravarty, Lach, and Blaug (14); and data for Tween 20 were calculated from antifungal concentrations reported by Aoki, *et al* (9).

in Myrj 52[6] can be employed to calculate the ratio of total to free preservative as a function of the concentration of Myrj 52 These data are illustrated in Fig 5 for comparison with the binding exhibited by Tween 80

The ratios of total to free paraben in the presence of Tween 20, shown in Fig 5, were estimated from inhibitory concentrations reported by Aoki, Kamata, Yoshioka, and Matsuzaki (9) These determinations were made in modified Sabouraud media, but the results seem to indicate that the binding of the parabens by Tween 20 would be quite similar to that of Tween 80 and Myrj 52

It is significant that the interaction of propylparaben and other relatively hydrophobic esters with Tween is much greater than the interaction of the methyl ester Thus, for a given concentration of surfactant, a much greater proportion of the total propylparaben will be in an inactive form than would be the case with the methyl ester. Aoki, *et al.* (9), noted that while the higher esters such as propyl and butyl were effective in lower concentration than the methyl ester in water, in the presence of surfactants this relationship is sometimes reversed. It should be possible to select the most suitable preservative for a given system, utilizing data such as those presented in Fig 5

[6] Myrj 52 is polyoxyl 40 stearate, Atlas Powder Co., Wilmington, Del

SUMMARY

1. The preservative activity of p-hydroxybenzoic acid esters in the presence of nonionic surfactants is primarily a function of the concentration of unbound preservative.
2. The p-hydroxybenzoates can be employed as efficient preservatives in the presence of nonionic surfactants, but the proper concentrations must be employed This study suggests that these concentrations can be estimated by multiplying the usual preservative concentration by the ratio of total to free paraben at any given surfactant concentration
3 Hydrophobic esters such as propyl and butyl are usually effective in lower concentrations than the methyl ester when employed in aqueous systems, but in the presence of surfactants of the polyoxyethylene type this relationship might sometimes be reversed.

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Interaction of Preservatives With Macromolecules III*

Parahydroxybenzoic Acid Esters in the Presence of Some Hydrophilic Polymers

By GRACE M. MIYAWAKI†, N. K. PATEL‡, and H. B. KOSTENBAUDER

The magnitude of any intermolecular association occurring in aqueous solution between methyl- and propylparaben and several hydrophilic polymers was determined. Parabens were found to interact to some extent with polyethylene glycol 4000, methylcellulose, polyvinylpyrrolidone, and gelatin. There was no evidence for a significant degree of interaction between the parabens and carboxymethylcellulose or tragacanth. The significance of these results relative to preservation of solutions containing these polymers is considered.

PREVIOUS STUDIES in this series (1, 2) have been concerned with the rather significant interaction of preservatives with nonionic surfactants of the polyoxyethylene type and the importance of considering such interactions when determining proper preservative concentrations. Several articles published in recent years indicate that some thought has been given to interactions of preservatives with macromolecules such as tragacanth (3, 4), methylcellulose (3, 5), carboxymethylcellulose (3), Irish moss (3), and polyethylene glycols (6). The purpose of the present investigation was to determine the extent of any such interactions which might occur between *p*-hydroxybenzoic acid esters and some representative macromolecules, and thus obtain an evaluation of the relative importance of such binding in determining required preservative concentrations.

Studies were performed on polyethylene glycol 4000, methylcellulose, polyvinylpyrrolidone, gelatin, carboxymethylcellulose, and tragacanth, using solubility and equilibrium dialysis techniques to detect any possible association between the parabens and the macromolecules.

EXPERIMENTAL

Reagents.—Recrystallized methyl *p*-hydroxybenzoate,[1] m. p. 128–128.5°; recrystallized propyl *p*-hydroxybenzoate,[2] m. p. 96–98°; polyethylene glycol 4000;[3] methylcellulose 15 c. p. s.;[4] carboxymethylcellulose, low viscosity;[5] tragacanth;[6] gelatin, derived from an acid-treated precursor;[7] and polyvinylpyrrolidone.[8] The concentrations of all macromolecular dispersions are expressed on a w/v basis and have been corrected for moisture content as determined by drying a sample of the material to constant weight.

Equilibrium Dialysis Method.—The equilibrium dialysis technique employed in the present study is essentially the same in principle as that used by Klotz, Walker, and Pivan (7) and Karush and Sonenberg (8) in investigating the binding action of proteins, and by Higuchi and co-workers (9, 10, 11) in their studies of the interaction between some macromolecules and pharmaceuticals. The experimental technique was identical to that used by Patel and Kostenbauder (1) in studying the interaction of parabens with Tween 80,[9] with the exception that the membrane employed in these studies was cellulose dialyzer tubing.[10]

The dialysis method was employed for the study of methylcellulose, polyvinylpyrrolidone, gelatin, carboxymethylcellulose, and tragacanth.[11] Twenty-five ml. of the polymer solution under study was placed in each cellophane bag. Each bag was then tightly tied and placed in a wide-mouth glass-stoppered bottle containing 25, 50, or 75 ml. of an aqueous paraben solution. The capped bottles were then agitated for fifteen hours at constant temperature. For the more viscous tragacanth dispersions a longer period of equilibration was required and the above method was modified in that the paraben was dissolved in the tragacanth dispersion and the bottles containing these samples were agitated for several days until constant assay results were obtained for the external solution. The method of assay and the treatment of data have been described previously (1).

The volume of the solution outside the membrane was measured, after equilibration, to permit correction of the polymer concentration in the event a volume change had occurred. Important volume changes were noted only in the carboxymethylcellulose solutions.

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This work was supported in part by a research grant from Heyden Newport Chemical Corp., New York.

† Chas Pfizer Fellow in Hospital Pharmacy, 1957–1958.

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[1] Methyl Parasept, purified, supplied through the courtesy of Heyden Newport Chemical Corp., New York

[2] Propyl Parasept, purified, supplied through the courtesy of Heyden Newport Chemical Corp , New York.

[3] "Carbowax" 4000, supplied through the courtesy of Union Carbide Chemicals Co , New York.

[4] Methocel 15 c. p. s , supplied through the courtesy of Dow Chemical Co., Midland, Mich

[5] CMC, 70 premium low, supplied through the courtesy of Hercules Powder Co , Wilmington, Del.

[6] S B. Penick & Co., New York.

[7] Pharmagel A, Pharmagel Corp., New York.

[8] Plasdone, supplied through the courtesy of Antara Chemicals, New York.

[9] Tween 80 is polyoxyethylene (20) sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

[10] A. H. Thomas, Philadelphia, Pa., average pore diameter 48 Ångström.

[11] Tragacanth contains a small fraction of dialyzable material; but any error introduced by neglecting this fraction is probably insignificant, particularly in view of the nature of the data obtained.

Solubility Method.—The solubility of methyl and propyl esters of *p* hydroxybenzoic acid was determined in the presence of varying concentrations of polyethylene glycol 4000 Excess quantities of paraben (0 25 Gm of the propyl ester, 1 25 Gm of the methyl ester) were placed in 125 ml glass stoppered bottles containing 50 ml of polyethylene glycol 4000 solution The bottles were agitated for twenty four hours at 30° The method of assay and treatment of data have been described previously (1)

RESULTS AND DISCUSSION

Polyethylene Glycol 4000—Lach, Ravel, and Blaug (6) determined the solubility of methyl and propyl *p* hydroxybenzoates in aqueous solutions containing up to 2% PEG 4000 and PEG 6000 In the present study the solubility determinations were extended to 10% w/v PEG 4000, as illustrated in Fig 1 In Fig 2 the solubility data have been plotted to show the ratio of total to free paraben as a function of PEG 4000 concentration As illustrated in a previous study (2), the ratio, *r*, shown in Fig 2, can be used as a first approximation in determining any increased quantity of preservative which might be required in the presence of the PEG For example, if it can be assumed that any interference with the activity of the preservative is due to binding of the macromolecule, and the macromolecule itself exhibits no antimicrobial activity, then in a solution of 5% PEG 4000 it might be expected that the concentration of methylparaben employed should be approximately 1 6 times the concentration which would be employed in the absence of the PEG While this approach is undoubtedly an oversimplification of actual conditions, it nevertheless provides some indication of the quantity of preservative which might be required

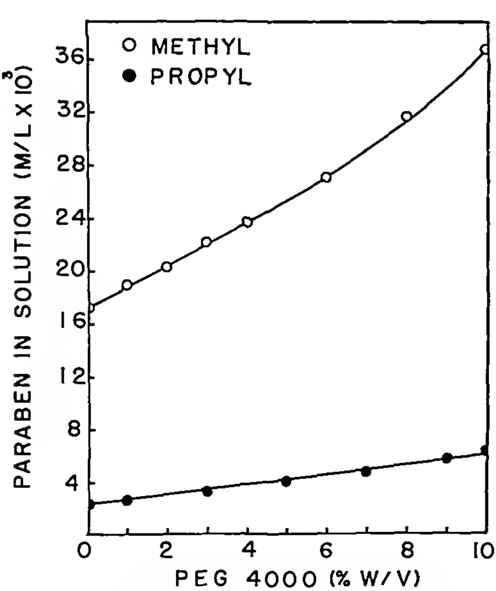

Fig 1—Solubility of methyl and propyl *p* hydroxybenzoate in aqueous solutions of polyethylene glycol 4000 at 30°

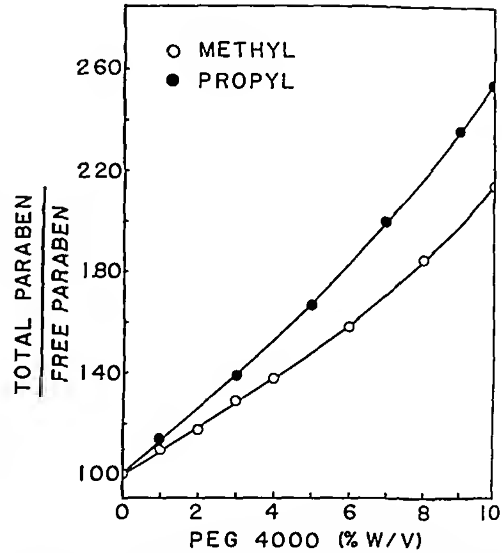

Fig 2—Ratio, *r*, of total paraben to free paraben as a function of polyethylene glycol 4000 concentration at 30°

The binding of parabens by PEG is of minor importance in comparison with the binding exhibited by polyoxyethylene surfactants (1, 2), and results reported by deNavarre and Bailey (12) suggest that in practice there is little or no interference with the preservative activity of methylparaben in the presence of 2% PEG 4000 This result is not surprising, since preservatives are usually employed in concentrations well in excess of the minimum inhibitory concentration, thus, binding a portion of the preservative might not reduce the effective concentration to a level which would permit microbial growth

Figure 2 indicates that the propyl ester is bound to a greater extent than the methyl ester, but the difference in binding affinity is considerably less than was observed for these two compounds in solutions of polyoxyethylene surfactants (2)

Methylcellulose—Tillman and Kuramoto (5) studied the solubility of several parabens in the presence of methylcellulose and obtained evidence which would suggest the existence of both soluble and insoluble complexes between the parabens and this polymer It is possible that the phenolic group of the paraben might interact with the ether groups of the methylcellulose in a manner analogous to the interaction with PEG

The dialysis method was employed in the present study to obtain an estimate of the degree of interaction in the concentration range in which the parabens might normally be employed Figure 3 illustrates the ratio of total to free paraben as a function of methylcellulose concentration While there is definite evidence that an interaction does occur, the ratio of total to free paraben indicates that in the concentrations in which methylcellulose might normally be employed it is unlikely that sufficient paraben would be bound to the macromolceule to necessitate the addition of supplementary quantities of paraben

Polyvinylpyrrolidone—The interaction of methyl and propyl *p* hydroxybenzoates with PVP was investigated by means of the dialysis technique The

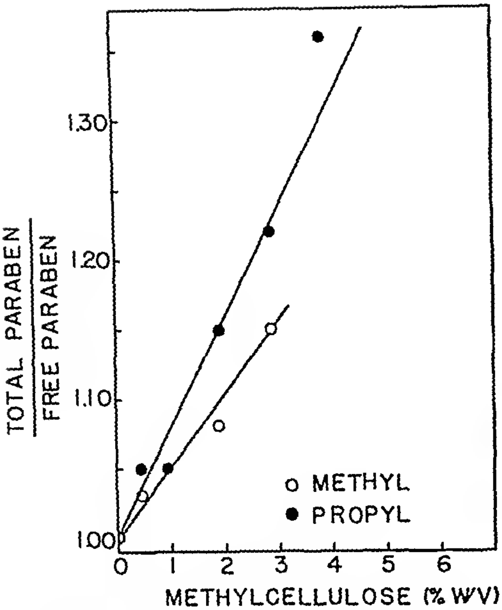

Fig. 3.—Binding of methyl and propyl p-hydroxybenzoate by methylcellulose at 30°. Total methylparaben in solution 6.41×10^{-3} M; total propylparaben in solution 1.23×10^{-3} M.

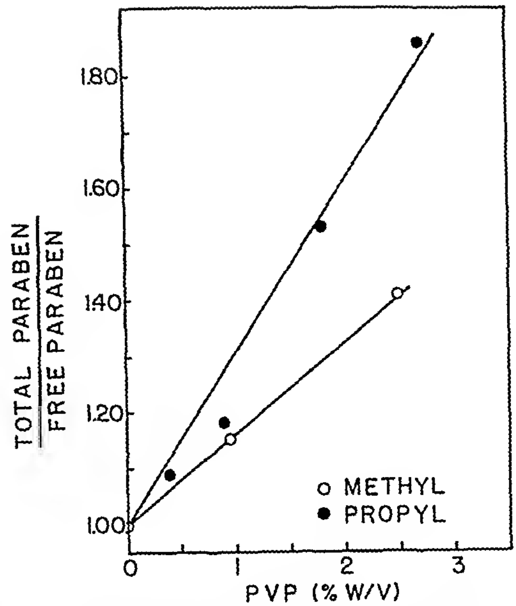

Fig. 4.—Binding of methyl and propyl p-hydroxybenzoate by polyvinylpyrrolidone. Data for methylparaben obtained at 27°, and data for propylparaben at 30°. Total methylparaben in solution 6.12×10^{-3} M; total propylparaben in solution 1.23×10^{-3} M.

ratio of total to free paraben as a function of PVP concentration is illustrated in Fig. 4.

PVP contains an amido group; and it has previously been demonstrated that compounds such as phenols, which are capable of acting as proton donors, tend to form complexes with amides (9, 13). Of the polymers included in this study PVP exhibited maximum binding of the parabens.

Gelatin.—The results of a dialysis study of methyl and propylparaben in the presence of gelatin provided evidence, as indicated in Fig. 5, for some degree of interaction. These results were obtained in unbuffered solutions at a pH of 4.3–4.4, using gelatin derived from an acid-treated precursor. The interaction of the paraben with the gelatin can probably be attributed to the presence of the amide-type groups in the gelatin.

The degree of binding observed in the presence of gelatin appears to be of relatively minor importance in determining required preservative concentrations.

Carboxymethylcellulose.—The possible interaction of CMC with methylparaben was investigated by means of the equilibrium dialysis method and the results are presented in Fig. 6. Extreme volume changes occurred in these solutions due to the osmotic activity of the CMC. To prevent these changes when attempting to investigate more concentrated CMC solutions it was necessary to add sodium chloride as a swamping electrolyte. In no case was there any detectable evidence of association between the paraben and the CMC.

Tragacanth.—Dialysis data for methylparaben in the presence of tragacanth are presented in Fig. 6. The results of these studies indicate that there is no significant binding of the preservative by the tragacanth. If increased quantities of the parabens should be required for effective preservation of tragacanth dispersions this increase must be attributed to some factor other than adsorption of the preservative. It is probable that tragacanth dispersions provide a more favorable medium for the growth of microorganisms; and, if so, it might be expected that the necessity for increased preservative concentrations would not be limited to any specific preservative.

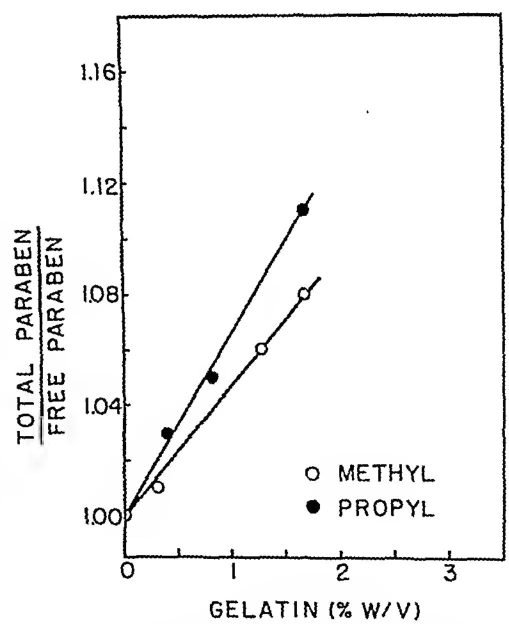

Fig. 5.—Binding of methyl and propyl p-hydroxybenzoate by gelatin at 30°. Total methylparaben in solution 9.80×10^{-3} M; total propylparaben in solution 1.85×10^{-3} M.

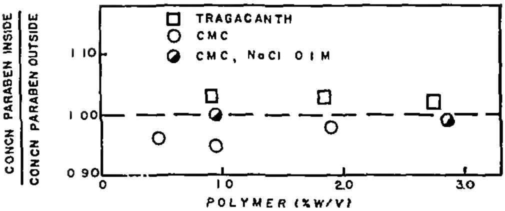

Fig 6—Ratio of the concentration of methylparaben inside the dialysis membrane to the concentration outside the membrane, in the presence of varying concentrations of tragacanth and carboxymethylcellulose at 30° Methylparaben concentration 6 60 × 10^{-3} to 9 88 × 10^{-3} *M*.

Table I presents a comparison of the degree of binding of parabens in the presence of 2% dispersions of the macromolecules studied Although data are not presented for propylparaben in CMC and tragacanth dispersions, the results would be expected to parallel those obtained with the methyl ester

TABLE I—COMPARATIVE BINDING OF PARABENS BY 2% POLYMER SOLUTIONS

Macromolecule 2% w/v	Total Methyl paraben Free, %	Total Propyl-paraben Free, %
Polyvinylpyrrolidone	78	64
Polyethylene glycol 4000	84	81
Methylcellulose	91	87
Gelatin	92	89
Carboxymethylcellulose	100	
Tragacanth	100	

SUMMARY

1. Methyl and propyl *p*-hydroxybenzoate were found to interact to some extent with polyethylene glycol, methylcellulose, polyvinylpyrrolidone, and gelatin. The magnitude of the association is considerably less than that observed previously with several nonionic surfactants.

2. There was no evidence for a significant degree of interaction of methylparaben with carboxymethylcellulose or tragacanth. Any increased quantity of paraben required to preserve tragacanth dispersions cannot be attributed to adsorption of the preservative by the gum, and thus should not be a phenomenon restricted to any specific preservative.

3 It would appear that for concentrations in which the polymers under investigation are usually employed, binding of the parabens would not be sufficient to prohibit their efficient application as preservatives.

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The Determination of Acetophenetidin in Tablet Mixtures: A Potentiometric Method*

By JOSEPH G. BALDINUS and IRVIN ROTHBERG

When acetophenetidin is hydrolyzed with acid, the resulting *p*-phenetidin can be titrated potentiometrically with sodium nitrite if potassium bromide is used as a catalyst to speed the diazotization reaction. This provides the basis for a simple, rapid, and accurate procedure for determining acetophenetidin. Aspirin, caffeine, salicylic acid, codeine, amphetamine, and amobarbital do not interfere with the titration.

RECENTLY, Falex (1) reported that *p*-phenetidin could be titrated with sodium nitrite using starch-iodide paper as an indicator This would be an attractive procedure for acetophenetidin, except for a number of disadvantages: the starch-iodide paper is insensitive to dilute solutions of sodium nitrite, the temperature must be controlled, colored solutions give trouble, the reaction with starch-iodide paper requires considerable time, and, finally, the use of an external indicator is simply not conducive to accuracy and precision.

In the work presented below, the diazotization reaction was speeded so that the titration of the *p*-phenetidin could be followed potentiometrically. The resulting procedure has proved considerably less troublesome than the existing methods for determining acetophenetidin (2–10).

* Received October 7, 1958, from the Smith Kline and French Laboratories, Philadelphia 1, Pa.

EXPERIMENTAL

Reagents.—A 0 05 *N* sodium nitrite solution which was prepared by dissolving approximately 3 5 Gm sodium nitrite in one liter of distilled water; sulfanilamide, U S P reference standard, which was used to standardize the nitrite solution; potassium bromide crystals; and a 10% aqueous solution of HCl

Apparatus.—Either a Photovolt model 110 or a Beckman model G pH meter; a platinum electrode which was used *versus* a calomel electrode; a timer, preferably one with a second hand; and a 25-ml buret

Procedure.—The tablets were ground to a fine powder and an amount of sample that contained about 160–180 mg. of acetophenetidin was refluxed for one to one and one-half hours with 50 ml of 10% aqueous HCl. The resulting solution was transferred quantitatively to a 250-ml beaker with the aid of 50 ml 10% aqueous HCl, 5 Gm of potassium bromide was added, and the solution was then titrated potentiometrically with sodium nitrite, using a magnetic stirrer for agitation At the beginning of the titration, the titrant was added rapidly, but near the end point, increments of 0 1 ml were added Since the potential drifted during the titration, the readings were recorded exactly one minute after each addition of titrant The inflection point was calculated by the method of Kolthoff and Laitinen (11)

A blank titration was also run on the sample prior to hydrolysis with acid. On all our samples, the blanks were negligible. Ordinarily, the blank would be nil unless *p*-phenetidin had somehow formed in the tablets This conceivably could happen with age or under unusual storage conditions For control work, the blank determination could probably be omitted altogether After subtraction of the blank, each ml of 0 05 *N* sodium nitrite consumed was equivalent to 8 955 mg acetophenetidin

In standardizing the sodium nitrite with sulfanilamide, it was not necessary to use potassium bromide However, the potentials were still recorded as above, one minute after the addition of each increment of titrant.

DISCUSSION AND RESULTS

Without the use of potassium bromide, a potentiometric end point could not be obtained when *p*-phenetidin was titrated with sodium nitrite Concentrations of potassium bromide ranging from 1–10% were tried, and the optimum concentration was found to be about 5% Below concentrations of 4%, the size of the end point seemed to diminish, above 5% no improvement was noted

Iodide has been reported (12) to be an even more effective catalyst for the diazotization of aromatic amines than bromide While this may be true at very low acidities, in our titrations the iodide was simply oxidized by the sodium nitrite

The dead stop method (13) using polarized platinum electrodes was also tried With potassium bromide, the end point could be detected; however, it was not nearly so pronounced or so reproducible, as was the potentiometric end point

Caffeine, aspirin, salicylic acid, and the common tablet excipients, such as starch, talc, magnesium stearate, calcium sulfate, and guar gum were checked for possible interference in the titration; however, no interference was found Codeine, amphetamine, and amobarbital, also, did not affect the titration.

In the blank titration, the acetophenetidin comes in contact with a 10% acid solution and might be expected to hydrolyze slightly Pure acetophenetidin, however, gave a negligible blank even after thirty minutes stirring in the acidic solution.

RESULTS

When pure acetophenetidin was analyzed by the above procedure, theoretical recoveries were obtained Results obtained on various tablet mixtures are reported in Table I.

TABLE I—ANALYSIS OF ACETOPHENETIDIN IN VARIOUS TABLETS

Sample	Label Declaration, gr /tablet	Found, gr /tablet
1[a]	5 00	4 94
2	5 00	5.02
3	5 00	5 00
4	5 00	4.95
5	5 00	4 97
6	5 00	4 94
7	5 00	5 00
8	5 00	4 96
9	5 00	4 95
10	5 00	4 98
11	5 00	5 02
12	5 00	5 02
13[b]	2 50	2 50
14[b]	2 50	2 52
15[c]	2 50	2 48
16[c]	2 50	2 50

[a] Samples 1–12 inclusive are APC tablets
[b] These tablets contain codeine sulfate, amphetamine sulfate, and aspirin in addition to the acetophenetidin
[c] Amphetamine sulfate, amobarbital, and aspirin are present in these samples

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Substituted α-Benzylphenethylamines*

By WILLIAM H. EDGERTON†, ROBERT L. HULL, and JAMES R. FISHER

A series of amines related to α-benzylphenethylamine was prepared. The preparation of the penicillin G salts of these amines was attempted. The most interesting compounds reported are the penicillin G salts of α-benzyl-N-(3-phenylpropyl)-phenethylamine and α-benzyl-N-(p-butoxybenzyl)-phenethylamine.

MUCH EFFORT in the pharmaceutical industry has been expended to obtain formulations of penicillin G derivatives which would be stable for extended periods and yet yield high blood levels upon oral administration. The most notable of the insoluble amine-penicillin salts prepared for this purpose has been benzathine penicillin G (1).

α,N-Dibenzylphenethylamine (Table I, 3)[1] was found to form a crystalline salt with penicillin G. A series of related amines was synthesized in order to select the most desirable penicillin salt for further study. *p*-Substitution of the N-benzyl moiety by various alkoxy, alkyl, or halogen groups gave crystalline penicillin salts of solubilities[2] ranging from 0.05 to 0.009%. Substitution of other groups such as nitro or acetamido at this position gave amorphous salts. *o*- or *m*-Substitution gave uniformly negative results. Replacement of N-benzyl by N-cyclohexylmethyl, N-hexadecyl, or N-furyl-methyl moieties gave bases which would not form crystalline salts. Methylation of I, 3 by the Clark-Eschweiler reaction destroyed its ability to form crystalline salts.

Crystalline penicillin salts of low solubility were obtained from amines prepared by replacement of the N-benzyl group by phenyl substituted straight chain alkanes. The most interesting member of this group was the α-benzyl-N-(3-phenylpropyl)-phenethylamine penicillin salt (Table IV, 2). The amine (Table I, 5) was prepared most readily by the reductive alkylation of α-benzylphenethyl amine by hydrocinnamaldehyde. When cinnamaldehyde was used in the alkylation, lower yields resulted due to the formation of considerable amounts of a by-product, N-(α-benzylphenethyl)-cinnamylamine. Lower yields were the result of alkylation by phenylpropyl bromide or chloride.

An alternate method of synthesis was developed, consisting of Meerwein-Ponndorf-Verley reduction of dibenzyl ketone to the carbinol which was reacted with hydrogen bromide to give α-benzylphenethyl bromide. Although the bromide could not be purified easily, the crude compound was condensed with appropriate amines to obtain compounds not obtained readily otherwise.

The conversion of dibenzalacetone to 1-phenethyl-3-phenyl-propylamine via the reduction of the oxime has been reported (2), but the procedure proved to be very tedious in our hands. The ketone was reduced catalytically (3) with 5% palladium-on-charcoal until two molar equivalents of hydrogen had been absorbed. The Leuckart reaction using formamide gave a 76% yield of the desired amine. 5-Phenyl-1-(4-phenylbutyl)-pentylamine was prepared in similar fashion from 1,9-diphenyl-1,3,6,8-nonatetraen-5-one via 1,9-diphenyl-5-nonanone. None of a series of alkylated derivatives of these amines formed crystalline penicillin salts.

The dry distillation of the calcium salt of α-naphthylacetic acid gave 1,3-di-(α-naphthyl)-2-propanone. A Leukart amination and subsequent alkylation gave the dinaphthyl analog of I, 3 which formed an amorphous penicillin salt.

Hydrogenation of α-benzylphenethylamine with ruthenium dioxide catalyst at medium pressures and elevated temperatures resulted in a good yield of α-cyclohexylmethylcyclohexylethylamine. Alkylated derivatives of this amine were only partially successful in forming penicillin salts.

The pKa values of representative members of the series were determined by electrometric titration, usually in 5% *n*-propanol (4) in an attempt to correlate a physical property of the amines with the character of their penicillin salts. Although the variation in basicity with structure was largely that expected, no correlation in these values with the crystallinity of the penicillin salts was apparent. In general, alternations of the parent skeleton of I, 3 which destroyed the symmetry of the amine resulted in loss of the crystallinity of its penicillin salt.

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[1] Compounds are designated by Table No. and entry No

[2] [illegible]rmined most conveniently by a [illegible] developed by Mr J A Sultza-[illegible] A method based on ultraviolet [illegible] agitation of a suspension of the salt gave erratic results.

TABLE I.—α-BENZYL-PHENETHYLAMINES

$$\begin{array}{c} \quad\quad NRR' \\ \quad\quad | \\ C_6H_5—CH_2—CH—CH_2C_6H_5 \end{array}$$

No	R	R′	M. P., °C	Yield, %	pK′a	Prepn	Formula	Analyses, % Carbon Calcd	Carbon Found	Hydrogen Calcd	Hydrogen Found
1	CH_3	CH_3	223	83		[a]	$C_{17}H_{21}N$ HCl	79 03	74 14	8 09	7 87
2	H	$C_{16}H_{33}$	107	54	5 4	B	$C_{31}H_{49}N$ HCl	78 85	78 41	10 68	10 87
3	H	$CH_2C_6H_5$	246	94	6 5	A, C	$C_{22}H_{23}N$ HCl	78 20	77 61	7 16	7 39
4	H	$C_2H_4C_6H_5$	188	64	6 7	A, B	$C_{23}H_{25}N$ HBr	69 70	69 61	6 61	6 72
5	H	$C_3H_6C_6H_5$	193	79	7 2	A, B	$C_{24}H_{27}N$ HCl	78 77	78 92	7 71	7 55
6	H	$CH_2—CH(C_5H_{11})—CH_2C_6H_5$	146	46		A[b]	$C_{29}H_{37}N$ HCl	79 87	80 30	8 78	8 58
7	H	CH_2-C_6H_{11}	247	65	6 95	A[b]	$C_{22}H_{29}N$ HCl	76 81	76 68	8 79	9 01
8	CH_3	$CH_2C_6H_5$	160	71		[a]	$C_{23}H_{25}N$ HCl	78 50	78 26	7 45	7 20
9	H	$CH_2C_6H_4$ p-CH_3	242	52		B	$C_{23}H_{25}N$ HCl	78 50	78 53	7 45	7 57
10	H	$CH_2C_6H_4$ p $CH(CH_3)_2$	211	50	6 55	A	$C_{25}H_{29}N$ HCl	79 02	79 02	7 94	8 20
11	H	$CH_2C_6H_4$-p-OCH_3	225	81		A	$C_{23}H_{25}NO$ HCl	75 08	75 19	7 12	6 82
12	H	$CH_2C_6H_4$-p-OC_2H_5	222	85		A	$C_{24}H_{27}NO$ HCl	75 47	75 93	7 39	7 49
13	H	$CH_2C_6H_4$ m OC_2H_5	139	67	6 4	A	$C_{24}H_{27}NO$ HCl	75 47	75 63	7 39	7 62
14	H	$CH_2C_6H_4$ o OC_2H_5	192	83	6 7	A	$C_{24}H_{27}NO$ HCl	75 47	76 10	7 39	7 58
15	H	$CH_2C_6H_4$-p-OC_3H_7	209	84		A	$C_{25}H_{29}NO$ HCl	75 83	75 74	7 64	7 60
16	H	$CH_2C_6H_4$ p $OCH(CH_3)_2$	232	80		A	$C_{25}H_{29}NO$ HCl	75 83	76 24	7 64	7 73
17	H	$CH_2C_6H_4$ p OC_4H_9	203	82	6 65	A	$C_{26}H_{31}NO$ HCl	76 16	76 34	7 87	7 89
18	H	$CH_2C_6H_4$-p-$OCH(CH_3)C_2H_5$	229	81		A	$C_{26}H_{31}NO$ HCl	76 16	76 14	7 87	8 12
19	H	$CH_2C_6H_4$-p OC_5H_{11}	199	82		A	$C_{27}H_{33}NO$ HCl	76 48	76 49	8 08	8 29
20	H	$CH_2C_6H_4$ o OC_5H_{11}	139	39		A	$C_{27}H_{33}NO$ HCl	76 48	76 30	8 08	8 30
21	H	$CH_2C_6H_4$ p-$OC_2H_4CH(CH_3)_2$	220	82		A	$C_{27}H_{33}NO$ HCl	76 48	76 77	8 08	8 16
22	H	$CH_2C_6H_4$-p-$OC_{10}H_{21}$	173	80		A	$C_{32}H_{43}NO$ HCl	77 78	77 80	8 98	8 93
23	H	$CH_2C_6H_4$-p-$OCH_2C_6H_5$	225	83	6 65	A	$C_{29}H_{29}NO$ HCl	78 44	78 62	6 81	6 89
24	H	$CH_2C_6H_4$ p OC_2H_4Cl	207	85		A	$C_{25}H_{28}ClNO$ HCl	69 76	69 69	6 79	6 89
25	H	$CH_2C_6H_4$ p-Cl	257	14	6 1	A	$C_{22}H_{22}ClN$ HCl	70 96	71 50	6 23	6 50
26	H	$CH_2C_6H_4$-p-NO_2[c]	268	27	7 7	B	$C_{22}H_{22}N_2O_2$HCl	69 01	69 21	6 06	5 95
27	H	$CH_2C_6H_4$-p-$NHCOCH_3$	271	94		A	$C_{24}H_{26}N_2O$ HCl	72 98	72 89	7 05	6 82
28	H	$CH_2C_6H_4$-p-$N(C_2H_5)_2$	199	48		A	$C_{26}H_{32}N_2$ 2HCl·$^1/_4H_2O$[d]	68 03	68 08	7 80	8 09
29	H	$CH_2C_6H_3$ m-OCH_3 p-OH	205	78		A	$C_{23}H_{25}NO_2$HCl	72 44	72 60	7 09	7 23
30	H	CH_2 α-C_4H_3O	189	80		A	$C_{20}H_{21}NO$ HCl	73 27	72 89	6 76	7 09
31	H	H	209		8 0		$C_{15}H_{17}N$ HCl	72 71	72 39	7 32	7 14

[a] These compounds were prepared by Clarke Eschweiler methylation
[b] The readily available unsaturated aldehydes were used in the reductive alkylation
[c] Catalytic reduction of the p nitro compound gave a solid which could not be purified
[d] This compound, after drying *in vacuo*, was observed to be hygroscopic

On the basis of low solubility in water, ease of crystallization, and preparation as well as commercial availability, the penicillin salts of α-benzyl-N-(p-butoxybenzyl)-phenethyl amine (Table IV, 9) and α-benzyl-N-(3-phenylpropyl)-phenethylamine (Table IV, 2) were chosen for further study. These salts produced blood levels considerably higher than those obtained with benzethine penicillin G in dogs following oral administration. Both of these compounds caused irritation when administered by injection. Many of the amines prepared proved to be potent local anesthetics.

EXPERIMENTAL[1]

α-Benzylphenethylamine was prepared by the method of Koelsch (5) Alkoxybenzaldehydes were prepared by the method of Stoermer and Wodarg as well as Weygand and Gabler (6) Several aldehydes not previously reported are p-decyloxybenzaldehyde (b p 173–176° at 1 mm), p-chloropropoxybenzaldehyde (b p 110–115° at 1 mm), m-amoxybenzaldehyde (b p 109° at 0 5 mm) and sec-butoxybenzaldehyde (b p 135–137° at 6 mm)

α-Benzylphenethylamines.—*Method A* —An equimolar mixture of amine and aldehyde in ethanol was heated at reflux for one hour Appropriate catalyst, usually platinum oxide or 5% palladium-on-charcoal, was added and the mixture hydrogenated at three atmospheres at room temperature to completion After filtration and evaporation *in vacuo*, the residue was dissolved in ether The ether extract was dried over potassium hydroxide, filtered, and saturated with dry hydrogen chloride gas The crystalline product was usually recrystallized from aqueous ethanol

Method B —The primary amine was heated with a slight excess of appropriate alkyl halide with or without sodium carbonate for ten to twenty hours The mixture was then filtered and the product isolated either as the hydrochloride or the free base.

Method C —The reduction of dibenzyl ketone by magnesium methanol (7) or catalytic hydrogenation gave poor returns of dibenzyl carbinol A 70% yield of the alcohol was obtained using aluminum isopropoxide in the Meerwein-Ponndorf-Verley reaction

[1] All melting points and boiling points are uncorrected

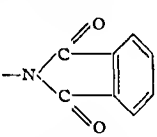
TABLE II —α-PHENYLALKYL-PHENYLALKYLAMINES

$$C_6H_5(CH_2)_X\text{—}\underset{\displaystyle\text{NHR}}{\overset{}{CH}}\text{—}(CH_2)_YC_6H_5$$

No	R	X	Y	M P, °C	Yield, %	pK'a	Prepn	Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd	Hydrogen Fouad
1	$CH_2C_6H_5$	2	0	210	81	6 8	A	$C_{22}H_{23}N$ HCl	78.20	78.29	7.16	7.25
2	H	2	2	160	83	8 8	[a]	$C_{17}H_{21}N$ HCl	74 02	73.92	8.04	8.02
3	$CH_2C_6H_5$	2	2	118	79	7 5	A	$C_{24}H_{27}N$ HCl	78 77	78 55	7.44	7.80
4	$C_2H_4C_6H_5$	2	2	159	91		B	$C_{25}H_{29}N$ HCl	79 02	78 87	7 96	8.11
5	$C_3H_6C_6H_5$	2	2	88	75		A	$C_{16}H_{31}N$ HCl $^1/_2H_2O$	77 48	77.35	8 25	8.25[b]
6	$CH_2C_6H_4$-p-OCH_3	2	2	147	92		A	$C_{25}H_{29}NO$ HCl	75 83	75 85	7 64	7.74
7	H	4	4	[c]	31		[d]	$C_{21}H_{29}N$	85 36	84 70	9 89	9 89
8	$CH_2C_6H_4$ p OCH_3	4	4	98	63		A	$C_{29}H_{37}NO$ HCl	77 04	76 81	8.47	8 80[e]

[a] Previously reported by Stuhmer and Kaupmann (2) [b] Calcd N, 3 47 Found N, 3 30 [c] B p 177–178° at 0 7 mm [d] See Experimental [e] Calcd N, 3 09 Found N, 3 19

TABLE III.—MISCELLANEOUS RELATED AMINES

No	Structure	M P	Yield	pK'a	Prepn	Formula	Analyses, % Carbon Calcd	Carbon Found	Hydrogea Calcd	Hydrogea Fouad
1	$(C_6H_{11}CH_2)_2CH$-$NHCH_2C_6H_5$	208	76	7 7	A	$C_{22}H_{35}N$ HCl	75 50	75 45	10 37	10.20
2	$(C_6H_{11}CH_2)_2CHNHC_3H_6C_6H_5$	120	40	7 8	A	$C_{24}H_{39}N$ HCl	76 25	76 84	10 67	10 43[a]
3	$(C_6H_{11}CH_2)_2CHNHCH_2C_6H_4$-$p$-$OCH_3$	150	53	7 8	A	$C_{23}H_{41}NO$ HCl	73 58	73 25	10 38	10.14
4	$(1\ C_{10}H_7CH_2)_2CHNHCH_2C_6H_5$	238	75		A	$C_{30}H_{27}N$ HCl	82 26	82 16	6 44	6 53
5	$(C_6H_5CH_2)_2CH$-NHC_2H_4- 	230	64		B	$C_{25}H_{29}N_2O_2$ HCl - $^1/_2H_2O$	69 84	70 12	6.47	6 33[b]
6	$(C_6H_5)_2CHCH_2NHCH_2C_6H_5$[c]	260	20	7 6	A	$C_{21}H_{21}N$ HCl	77 87	77 62	7 01	6.86
7	$(C_6H_5)_2CHCH_2NHCH_2C_6H_4$ p-OC_5H_{11}	217	62		A	$C_{26}H_{31}NO$ HCl	76 17	76 23	7 87	8 00
8	$[(C_6H_5CH_2)_2CHNHCH_2C_6H_4O]_2$-$C_3H_6$	238	46	..	A	$C_{42}H_{52}N_2O_2$ 2HCl	75 28	75.39	7 26	6 98

[a] Calcd N, 3 71 Found N, 3 55 [b] Calcd · N, 6 52 Found N, 6 51 [c] The primary base was prepared by the reduction of diphenylacetonitrile by the method of Benoit, G , Delavigne, R , and Eliopoulo, F , *Ann pharm. franç.*, 10, 181 (1952).

Dry hydrogen bromide gas was passed into 75 Gm (0.35 mole) of dibenzylcarbinol at 100–120° until the gas was no longer absorbed The layers were separated. The bromide layer was washed with water and sodium bisulfite solution After drying over anhydrous calcium chloride, the bromide was distilled; 60 Gm. (62%), b. p 140–142° at 1 mm.

A solution of 19 Gm. (0.082 mole) of once distilled α-benzylphenethyl bromide and 9 Gm. (0 084 mole) of benzyl amine in 150 ml. of absolute ethanol was heated at reflux for fourteen hours Upon cooling, 9 Gm. (32%) of white crystals, m. p 268–269°, was obtained This solid was converted to the hydrochloride and proved identical to I, 3.

Penicillin salts.—A solution of one equivalent of potassium penicillin G in a minimum of water was added, dropwise, with stirring to a solution of the hydrochloride salt of the α-benzylphenethylamine in a water-miscible solvent such as alcohol or N,N-dimethylformamide The mixture was then cooled to separate the crystalline penicillin salt The product was usually washed well with water and analyzed after thorough drying. Several were recrystallized from aqueous ethanol at room temperature.

α - Benzyl - N - (3 - phenylpropyl) - phenethylamine (Table I, 5).—Recrystallization of the hydrochloride mixture obtained from the reductive alkylation of α-benzylphenethylamine by cinnamaldehyde (*Method A*) gave considerable quantities of a white crystalline by-product, m p 245–246°. A satisfactory analytical sample could not be obtained by repeated crystallization from various alcohols.

Anal.—Calcd. for $C_{24}H_{25}N$ HCl: C, 79.21; H, 7.20. Found: C, 78.60; H, 7.59.

The hydrochloride was neutralized by shaking in ether-sodium hydroxide solution until clear. The ether extract was separated, dried, and concentrated. Distillation of the residue gave a colorless liquid with a distinctive amine odor, b p. 172–173° at 0.5 mm.

Anal.—Calcd for $C_{24}H_{25}N$: C, 88 03; H, 7.70; N, 4.28. Found: C, 88.18; H, 7 73; N, 4.31.

This liquid gave positive bromine and potassium permanganate tests for unsaturation and appeared to be N-(α-benzylphenethyl)-cinnamylamine.

Another preparation of I, 5 using hydrocinnamaldehyde as alkylating agent gave a 62% yield of saturated base, b p 200–201° at 0.8 mm. A forerun of unreacted aldehyde was also collected.

TABLE IV.—PENICILLIN SALTS

No	Amine	M. P.	Yield	Solubility, %	Potency[a] u./mg. Calcd	Potency[a] u./mg. Found	Analysis[b] Carbon Calcd	Carbon Found	Hydrogen Calcd	Hydrogen Found	Nitrogen Calcd	Nitrogen Found
1	I, 3	121	94	0.076	934	907	71.78	71.28	6.50	6.59	6.61	6.82
2	I, 5	119	91	0.020	875	862	70.46	70.89	6.95	6.95		
3	I, 9	116	95	0.060			72.08	71.26	6.66	6.93		
4	I, 10	138	95	0.022			72.64	72.59	6.79	6.88		
5	I, 11	116	90	0.056	892	755	70.35	69.16	6.51	6.74	6.31	6.24
6	I, 12	130	95	0.047			70.66	71.12	6.67	6.34	6.18	6.34
7	I, 15	135	88	0.038			70.97	70.73	6.83	7.05	6.06	6.08
8	I, 16	143	96	0.033			70.97	70.93	6.83	6.99	6.06	5.93
9	I, 17	134	95	0.017	839	831	71.26	71.25	6.98	6.82	5.94	6.18
10	I, 18	143	90	0.026			71.26	71.86	6.98	7.39	5.94	5.49
11	I, 19	135	88	0.012	823	731	71.53	71.39	7.12	7.19	5.82	5.99
12	I, 21	135	85	0.019			71.53	71.38	7.12	7.27	5.82	5.70
13	I, 22	110	91	0.013			72.78	73.52	7.76	8.02	5.31	5.07
14	I, 23	141	95	0.009	800	758	72.75	72.35	6.51	6.51	5.66	5.51
15	I, 24	130	75	0.020	816	797	67.61	67.70	6.37	5.50	5.77	5.57
16	I, 25	128	80	0.066			68.09	68.21	6.01	6.18	6.27	6.10
17	III, 1	152	55	0.031			70.40	70.22	8.25	8.36		

[a] Supplied by Dr. John Ehrlich and Mrs. Margaret Galbraith.
[b] The penicillin salts often were analyzed without recrystallization. Many were noted to contain water by Victor Meyer assay.

Anal.—Calcd. for $C_{24}H_{27}N$: C, 87.49; H, 8.26. Found: C, 87.40; H, 8.32.

Several other salts of I, 5 were prepared by shaking the base and appropriate acid in water for several hours. The salts were isolated, analyzed, and characterized, acetate (m. p. 102°), sulfamate (m. p. 159°), lactate (m. p. 70°), nitrate (m. p. 137°), and oxalate (m. p. 206°).

1-Phenethyl-3-phenylpropylamine (Table II, 2).—The Leuckart reaction on 1,5-diphenyl-3-pentanone (3) gave a 76% yield of colorless liquid, b. p. 162–173° at 1.4–1.8 mm. A sample was converted to the hydrochloride (2), m. p. 157–160°.

5-Phenyl-1-(4-phenylbutyl)-pentylamine (Table II, 7).—The Leuckart reaction on 1,9-diphenyl-5-nonanone[4] gave a 31% yield of yellow liquid, b. p. 177–178° at 0.7 mm. This slightly impure amine was used for further alkylations.

1,3-Di(α-naphthyl)-2-propanone.—A solution of 38.8 Gm. (0.97 mole) of sodium hydroxide pellets in 1,500 ml. of water along with 170.7 Gm. (0.97 mole) of crude α-naphthylacetic acid was prepared with stirring. After filtration, a solution of 71.3 Gm. (0.485 mole) of calcium chloride dihydrate in 500 ml. of water was added. The mixture was cooled and 155 Gm. (78%) of the calcium salt was separated by filtration.

The dried salt (155 Gm., 0.378 mole) was powdered and dry distilled to yield a liquid, b. p. 220–280° at 2–5 mm. which solidified upon cooling. The solid was recrystallized twice from ethanol to give 55 Gm. (47%) of fine needles, m. p. 110–111°.

Anal.—Calcd. for $C_{23}H_{18}O$: C, 89.00; H, 5.85. Found: C, 89.22; H, 6.04.

α-(α-Naphthylmethyl)-α-naphthylethylamine.—The Leuckart reaction on the ketone gave a 50% yield of colorless crystals, from 70% ethanol; m. p. 105–106°. Hydrolysis of the intermediate formyl derivative was accomplished by refluxing in 100 ml. of concentrated hydrochloric acid and 200 ml. of ethanol.

Anal.—Calcd. for $C_{23}H_{21}N$: C, 88.71; H, 6.80. Found: C, 88.74; H, 6.95.

[4] A 92% yield, b. p. 193–196° at 0.7–0.8 mm., from the catalytic reduction of 1,9-diphenyl-1,3,6,8-nonatetraen-5-one.

α-Cyclohexylmethylcyclohexylethylamine.—A suspension of 211 Gm. (1.0 mole) of α-benzylphenethylamine in 500 ml. of purified dioxane with 5 Gm. ruthenium dioxide (8) was hydrogenated at 130–140° at 1,200–1,400 p. s. i. for eight hours. The catalyst was removed by filtration and the solvent evaporated *in vacuo*. The dark residue was distilled to give 158 Gm. (72%) of colorless liquid, b. p. 125–126° at 1.4–1.5 mm. A sample was refractionated, b. p. 98° at 0.5 mm.

Anal.—Calcd. for $C_{15}H_{29}N$: C, 80.65; H, 13.08. Found: C, 81.21; H, 12.23.

The hydrochloride was prepared with hydrogen chloride gas in ether. The white solid was recrystallized from water, m. p. 172–173°.

Anal.—Calcd. for $C_{15}H_{29}NHCl$: C, 69.33; H, 11.64; N, 5.39; Cl, 13.65. Found: C, 69.08; H, 11.79; N, 5.32; Cl, 13.65.

N, 2-Dibenzyl-3-phenylpropylamine (Table III, 6).—A solution of 4.0 Gm. (0.018 mole) of α-benzylcinnamaldehyde (9) and 1.9 Gm. (0.018 mole) of benzylamine in 50 ml. of ethanol was heated at reflux for one hour. The solution was hydrogenated with 5% palladium-on-charcoal at room temperature and three atmospheres. After filtration and evaporation *in vacuo*, the residue was dissolved in ether and saturated with dry hydrogen chloride gas to give, after recrystallization from methanol, 2.2 Gm. (35%) of white crystals, m. p. 144–146°.

Anal.—Calcd. for $C_{23}H_{26}ClN$: C, 78.50; H, 7.45. Found: C, 78.11; H, 7.46.

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An Improved Method for the Determination of Lanthionine*

By M. X. SULLIVAN and CHESTER F. MIJAL†

SUBSEQUENT to the discovery of cystine by Wollaston (1) in 1810 in a urinary calculus, a great deal of work was done as to its nature and occurrence. Baudrimont and Malaguti (2) found that cystine contained sulfur, which previous workers had missed. Then Morner (3) isolated cystine from the hydrochloric acid hydrolyzate of horn. Subsequently, cystine was found widespread in proteins by a host of investigators.

Dependent on the method of purification of the particular protein, the procedure of isolation of cystine, and its determination, great variation was found in the cystine content of individual proteins. Hoffman (4), in 1925, reported that hair treated with hot sodium carbonate (1 to 4 per cent) for a short time lost about 24 per cent of its sulfur. The treated hair was identical in appearance with that which had not been treated. However, although the treated hair retained about 75 per cent of its original sulfur, no cystine could be isolated from an acid hydrolyzate. About the same time Sullivan (5), employing his highly specific test for cystine, found that grain-curd casein, never in contact with alkali, had a higher cystine content than Hammarsten casein because the latter had gone through a sodium carbonate treatment. Later, Sullivan and Hess (6), using the Sullivan cystine test, found that treating various proteins with dilute alkali lowered the cystine content, and they (7) also found that treatment of insulin with pyridine lowered the cystine content of the hydrolyzed insulin. The pyridine treatment apparently did not affect the physiological unitage.

All our work pointed to the conclusion that treatment with weak alkali made changes in the nature of the protein, especially as to the amount of cystine found in an acid hydrolyzate.

In the meantime, Kuster and Irion (8) reported the isolation of a thioether from the acid hydrolyzate of wool heated with sodium sulfide. Horn, Jones, and Ringel (9) were unable to repeat this isolation, but did succeed in isolating a thioether by boiling wool in a 2 per cent sodium carbonate solution, and hydrolyzing the treated wool with 20 per cent hydrochloric acid solution. On concentrating the hydrolyzate, dissolving the concentrate in absolute ethyl alcohol, and adding pyridine to the alcohol extract, they obtained a certain amount of a thioether which they named "lanthionine" and found to be structurally ($COOH\text{-}CHNH_2\text{-}CH_2S\text{-}CH_2\ CHNH_2\text{-}COOH$)—a symmetrical thioether. The structure was confirmed by du Vigneaud and Brown (10) by synthesis of *meso*-lanthionine from *l*-cysteine and methyl *d,l*-α-amino-β-chloropropionate hydrochloride in strongly alkaline solution. Horn and Jones (11) then isolated lanthionine from human hair, chicken feathers, and lactalbumin, after treatment with sodium carbonate, and du Vigneaud and co-workers (12) found that amorphous insulin, similarly treated, yielded lanthionine. Others who have worked on the formation of lanthionine in a detailed, comprehensive way are Cuthbertson and Phillips (13) and Lindley and Phillips (14).

Lanthionine resembles methionine in that it is a thioether. Baernstein (15) had shown that hydriodic acid containing KH_2PO_2 (potassium hypophosphite) converts methionine into the thiolactone of homocysteine,

$$CH_3SCH_2CH_2CHNH_2COOH + HI = \underbrace{CH_2CH_2CHNH_2C}_{\text{—S—}} = O$$

Later, Hess and Sullivan (16) considered that one mole of lanthionine heated with hydriodic acid should yield 1 mole of cysteine, and developed a method for the quantitative determination of lanthionine. Lanthionine plus a 57 per cent solution of hydriodic acid, reinforced with potassium hypophosphite (KH_2PO_2) to prevent liberation of iodine, was hydrolyzed at 135–140° for four hours under a steady stream of nitrogen. A quantitative splitting resulted, with rupture of the carbon-sulfur bond of lanthionine and with the formation of cysteine.

The Sullivan (15) procedure for the estimation of cysteine was then applied. Lanthionine, as such, does not react in the Sullivan cysteine or cystine reaction, hence, if either of the latter compounds is present, it could be determined first, after hydrochloric acid hydrolysis, and then

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again, following hydrolysis with hydriodic acid, which yields only cysteine. The amount of lanthionine present would be measured by the increase in cysteine found after the hydriodic acid hydrolysis (*i.e.*, hydriodic acid hydrolysis minus hydrochloric acid hydrolysis). Multiplying the figure for the excess cysteine by 1.72 gives the amount of lanthionine.

The procedure of Hess and Sullivan for determining lanthionine is rather long, involves two separate hydrolyzates, and requires a special hydriodic acid. Accordingly, an investigation was made as to the possibility of splitting the lanthionine by cyanogen bromide, since the latter had been used by Cahours (17) to split such thioethers as dimethyl sulfide (CH_3SCH_3) by heating the thioether with solid cyanogen bromide in a sealed tube. Cahours considered that the reaction products were methyl thiocyanate (CH_3SCN) and a bromide of trimethyl sulfine [$(CH_3)_3SBr$]. Later, von Braun and Engelbertz (18) reported that the reaction is:

$$(CH_3)_2S + BrCN = CH_3SCN + CH_3Br$$

and that, as an intermediary product, [$(CH_3)_2CNSBr$] may occur.

In the light of the work by Cahours and especially by von Braun and Engelbertz, Sullivan and Folk (19) studied the action of cyanogen bromide on lanthionine isolated from hair by the procedure of Horn, Jones, and Ringel (9). Sullivan and Folk found that lanthionine, heated with excess sodium cyanide and cyanogen bromide, would react in the Sullivan cysteine reaction as cysteine does. Lanthionine treated with sodium cyanide or cyanogen bromide (*separately*) does not give the Sullivan cysteine reaction. Also, when cysteine or cystine is similarly heated with sodium cyanide and cyanogen bromide, no evidence of cysteine could be obtained. The free amino acids are either destroyed by the procedure or are changed to some compound not reacting as cysteine does. The Sullivan and Folk (19) procedure was offered as a test for lanthionine.

However, the procedure of alternately heating the lanthionine-sodium cyanide-cyanogen bromide mixture in an open tube is somewhat tedious and requires care in manipulation. Accordingly, we sought an easier method. Such a method was devised and is herein given.

EXPERIMENTAL

Procedure.—To 5 ml. of a 0.1 *N* hydrochloric acid solution containing 0.05–0.5 mg. of lanthionine in a 20 x 150 mm. test tube, add 1 ml. of 5% sodium cyanide solution. Shake gently and add 1 ml. of cyanogen bromide.[1] Stopper the test tube and heat for five minutes in a water bath held at 70°. Cool in cold water. To the cooled solution add 1 ml. of freshly prepared 0.5% aqueous solution of sodium 1,2-naphthoquinone-4-sulfonate. Shake the test tube for forty seconds and add 5 ml. of an alkaline sodium sulfite solution (10% of sodium sulfite in 0.5 *N* sodium hydroxide), stopper the tube tightly, and place it in the 70° water bath for fifteen minutes. Then cool in cold water and add 1 ml. of freshly prepared 2% sodium hydrosulfite ($Na_2S_2O_4$) in 0.5 *N* sodium hydroxide. Read the density of the color in the Klett-Summerson photoelectric colorimeter with the aid of the green filter (filter 54).

Preparation of a Standard Curve.—The precision and reproducibility of the quantitative lanthionine procedure as outlined above were studied. Ten duplicate samples of lanthionine solution of each concentration shown in Table I were run. The standard deviation and percentage standard deviation were calculated. The standard curve is shown in Fig. 1.

The reddish color produced fades quite rapidly, as may be seen in Fig. 2. Accordingly, after the addition of the sodium hydrosulfite, the interval of elapsed time before reading the absorbance in the Klett-Summerson colorimeter should be the same for all tubes.

TABLE I.

mg.	Av. *Reading*	Sum *Diff.*[2]	S. D.	S. D. *mg.*
0.05	31	29	1.78	±0.002
0.1	60.3	50	2.36	±0.004
0.2	121.2	161	4.31	±0.006
0.4	240.6	264	5.41	±0.009

Determination of Lanthionine Produced in Wool.—As a check on the method, a sample of white wool of unknown previous treatment (but possibly bleached) was employed. Thus 1 Gm. of the wool in question was boiled for thirty minutes with 200 ml. of a 2% solution of sodium carbonate. The treated wool was filtered on a sintered-glass funnel and washed with 200 ml. of distilled water. After being air-dried, the wool was hydrolyzed with 50 ml. of 20% hydrochloric acid solution until it no longer gave a biuret test. An equal volume of distilled water was added, and the solution (which was slightly colored) was treated with an iron-free Norit, boiled for two minutes, and filtered on a small Büchner funnel. The Norit was washed with 20 ml. of 0.1 *N* hydrochloric acid, and the washings were added to the main filtrate. The pH of the clear, colorless solution was adjusted to 3.5 with 5 *N* sodium hydroxide added dropwise with stirring, and the solution was transferred quantitatively to a 200-ml. volumetric flask and brought to volume by addition of 0.1 *N* hydrochloric acid.

The quantitative lanthionine determination was made by taking separately (*a*) a reagent blank (5 ml. of 0.1 *N* hydrochloric acid), (*b*) a hydrolyzate blank (1 ml. of the wool hydrolyzate plus 4 ml. of 0.1 *N* hydrochloric acid); (*c*) a standard control (2 ml. of standard lanthionine solution containing 0.2 mg. of lanthionine plus 3 ml. of 0.1 *N* hydrochloric acid);

[1] The cyanogen bromide is prepared by adding a 5% solution of sodium cyanide dropwise to a saturated, aqueous solution of bromine until a colorless solution results.

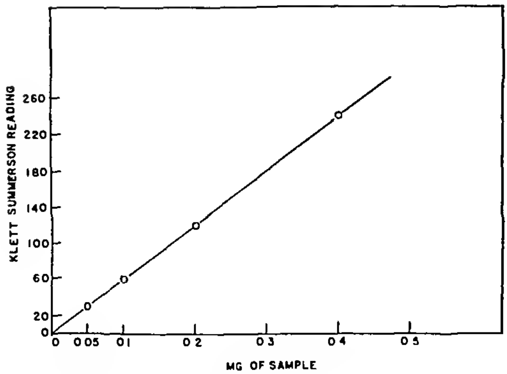


Fig 1 —Standard curve, average of 10 duplicate samples

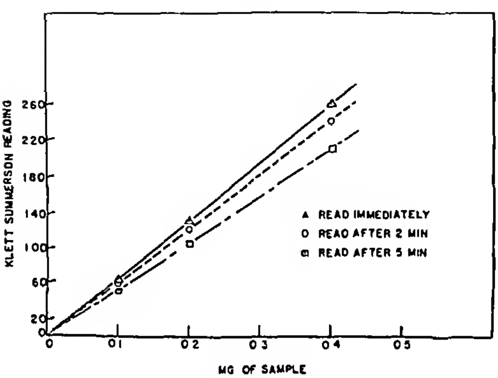


Fig 2.—Fading of color with time

and (*d*) the hydrolyzate (1 ml of the wool hydrolyzate plus 4 ml of 0 1 *N* hydrochloric acid)

To each of the four test tubes was added 1 ml of 5% aqueous sodium cyanide solution, followed by 1 ml of the cyanogen bromide solution The tubes were stoppered, kept in the water bath at 70° for five minutes, and then cooled to 15° in cold running water Successively, to each tube (with the exception of the hydrolyzate blank where water was substituted) was added 1 ml of sodium 1,2-naphthoquinone-4-sulfonate solution The test tube was stoppered and shaken for forty seconds Then, 5 ml. of sodium sulfite solution was added to each tube and, after being restoppered, the tubes were placed in the water bath, kept at 70° for fifteen minutes, and cooled to 15° In succession, one ml of the sodium hydrosulfite ($Na_2S_2O_4$) solution was added and, after shaking, the absorbance was read immediately

The following readings were obtained: (*a*) reagent blank 21, (*b*) hydrolyzate blank 10, (*c*) standard control 182, and (*d*) hydrolyzate 97.

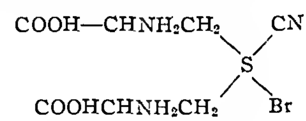
The calculations are: (*a*) standard control minus the reagent blank equals 161, and (*b*) the hydrolyzate minus the sum of the hydrolyzate blank and the reagent blank equals 66 These results indicate the presence of 0 082 mg of lanthionine per ml of the wool hydrolyzate, or 1 64% of lanthionine in this sample of alkali-treated wool.

DISCUSSION

The new procedure presented herein is a better colorimetric method for the quantitative determination of lanthionine, since it is performed more easily and obeys Beer's law over a wide range. It is somewhat different from that of Sullivan and Folk who, by strong heating, split the carbon-sulfur bond with the formation of a free sulfhydryl group (-SH), as shown by a positive nitroprusside test and by the cysteine estimated by the Sullivan cysteine reaction performed at room temperature. In the new reaction, no sulfhydryl (-SH) group is brought into play since the nitroprusside reaction is negative. Also, whereas Sullivan and Folk developed a color reaction at room temperature, the new procedure gives no color at room temperature on addition of the sodium cyanide, naphthoquinone derivative, and alkaline sodium sulfite solution In short, the color only develops, with these reagents, on heating at 70°

The specificity of the new lanthionine reaction is good. Related thioethers, such as thiodiglycolic acid ($COOHCH_2SCH_2COOH$), methionine (CH_3S-CH_2-CH_2CHNH_2COOH), and cystathionine ($COOH$-$CHNH_2CH_2CH_2SCH_2CHNH_2COOH$), treated with sodium cyanide and cyanogen bromide, gave no color Free cysteine and free cystine are oxidized at once by cyanogen bromide and no longer give a nitroprusside reaction with or without sodium cyanide and became negative in the regular Sullivan reactions for cysteine or cystine

Based on the work of von Braun and Engelbertz (18), it is possible that, in the lanthionine treatment, there is first formed a complex such as:



which split into $COOH$-$CHNH_2CH_2Br$ and $COOH$-$CHNH_2CH_2SCN$ The RSCN complex, on heating to 70° with sodium cyanide, the naphthoquinone derivative, sodium sulfite, and alkali, would be converted to a disulfide as suggested by the findings of Kaufmann (20) in the ease of thiocyanates of fatty acids However, the full explanation of the lanthionine reaction has not been ascertained

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Amine Derivatives of Cyanuric Chloride IV*

Catalytic Hydrogenolysis of Aromatically-Bound Chlorine

By WILLIAM O. FOYE and MELVIN H. WEINSWIG†

A method has been found whereby aromatically-bound chlorine may be reduced from cyanuric chloride derivatives. This involves catalytic hydrogenolysis with 10 per cent palladium-on-charcoal at atmospheric pressure. A series of mono- and diamino-*s*-triazines was prepared by this procedure without rupture of the *s*-triazine or other aromatic rings. Two of the diamino-*s*-triazines caused decreases of blood glucose in rats but showed no diuretic activity in dogs.

Cyanuric chloride is a convenient intermediate for the preparation of mono-, di-, or tri-substituted *s*-triazines, since stepwise replacement of the three chlorine atoms is possible (1). If a method were available where the unreacted chlorine atoms could be removed from the monoaminodichloro or diaminomonochloro derivatives so obtained, a simple means of preparing monoamino-*s*-triazines and diamino-*s*-triazines, or formoguanamines, would be available. Lipschitz and Hadidian (2), Clauder and Bulcsu (3), and Shapiro, *et al.* (4), have shown formoguanamine and its derivatives to be powerful diuretics. Their compounds, however, were obtained by rather tedious cyclization procedures which were unsuitable for preparing a number of triazine derivatives including the aminopyridyl and aminothiazolyl. Modest (5) has also depended upon a cyclization procedure to obtain aminodihydro-*s*-triazines which showed antivitamin, antimalarial, and antitumor activities.

Various attempts have been made to remove the chlorine atoms from cyanuric chloride derivatives to achieve a more convenient synthesis of mono- and disubstituted-*s*-triazines. Hirt, Nidecker, and Berchtold (6) reported the reduction in very small yields of 2-alkylamino-4,6-dichloro-*s*-triazines to 2-alkylamino-*s*-triazines using a palladium-on-charcoal catalyst. This is the only instance in the literature of the successful catalytic reduction of an *s*-triazine. Grundmann, *et al.* (7), and Burger and Hornbaker (8) were unable to hydrogenolyze the chlorines of cyanuric chloride using nickel and noble metal catalysts. The latter found also that the reduction of cyanuric chloride with lithium aluminum hydride gave only 2-dimethylamino-4,6-dichloro-*s*-triazine, which they rationalized as occurring through hydrogenolysis of the ring to form dimethylamine which then reacts with unchanged cyanuric chloride. Chafetz (9) later supported this hypothesis by isolating dimethylamine from this reaction. Grundmann (10) has explained the failures of catalytic hydrogenation attempts by showing that *s*-triazine is a powerful noble metal catalyst poison.

The catalyst selected for the present attempt toward removal of ring-halogen from aminochloro-*s*-triazines by hydrogen was a commercially prepared 10 per cent palladium-on-charcoal catalyst previously found suitable for reduction of amino acid chlorides to amino aldehydes (11). It was successful in catalyzing the removal of chlorine from cyanuric chloride derivatives with out rupture of the *s*-triazine ring, and the yields of amino-*s*-triazines obtained ranged from 28 to 82 per cent. The reactions were carried out at atmospheric pressure and temperatures of 60–80° by bubbling hydrogen through a suspension of the catalyst and either a solution or suspension of the chloro-*s*-triazine. The hydrogen chloride formed was bound by the amino-*s*-triazine, and excess hydrogen chloride could be titrated in the evolved gases. That other aromatically-bound halogen is removed by this procedure is shown by the loss of the chloro group from the *p*-chloroanilino substituents. The physical properties of the products obtained by this procedure are recorded in Table I.

Some difficulties with elemental analyses were encountered with these compounds as has been noted with other *s*-triazine derivatives (12). In these cases, which are probably due to depolymerization to cyanogen which reacts with the copper oxide in the combustion tube, analysis for ionizable chloride by the Volhard procedure generally gave results approximating the theoretical. Water determinations by loss of weight

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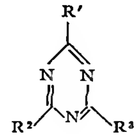
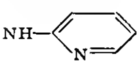
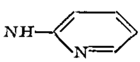
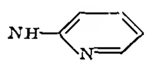
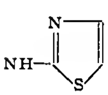
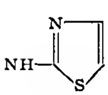
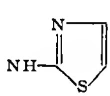
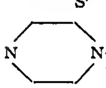
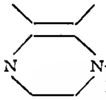
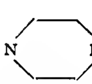
TABLE I.—AMINO-*s*-TRIAZINES



R'	R²	R³	Yield, %	M p [a], °C	Formula	Analyses, % Calcd		Found[b]
NH_2 HCl	NH_2	H	44	>300	$C_3H_6N_5Cl$	C	24 41	24 46
						H.	4 10	4 25
NH—$(CH_2)_2$—NH—$CO_2C_2H_5$ HCl	NH—$(CH_2)_2$—NH—$CO_2C_2H_5$	H	82	113–116	$C_{13}H_{24}N_7O_4Cl$ $2H_2O$	C	38 25	37 81
						H	6 93	6 87
						H_2O	8 7	8 6[c]
NH—C_6H_5 HCl[e]	H	H	30	250–252	$C_9H_8N_4Cl$	Cl	17 02	16 95[d]
NH—C_6H_5 HCl[e]	NH—C_6H_6 HCl	H	28	>300	$C_{16}H_{15}N_5Cl_2$	Cl	21 13	20 84
NH_2 HCl[e]	NH—C_6H_5 HCl	H	74	170–173	$C_9H_{11}N_5Cl_2$	Cl	27 54	27 11
 2HCl	H	H	35	238–241	$C_8H_9N_5Cl_2$ $4H_2O$	C	30 92	30 19
						H	5 35	5 35
						H_2O	22 6	22 2
 HCl		H	32	219–220	$C_{13}H_{12}N_7Cl$ $4H_2O$	C	41 71	40 09
						H	5 71	5 62
						Cl	9 52	9 47
						H_2O	19 3	19 8
 HCl	H	H	47	>300	$C_6H_6N_5SCl$	Cl	16 51	16 45
 HCl		H	33	>300	$C_9H_8N_7S_2Cl$	Cl.	11 18	10 99
 N—$CO_2C_2H_5$ HCl	H	H	77	220–222	$C_{10}H_{16}N_5O_2Cl$	C	43 92	43 91
						H	5 87	6 22
 N—CO.C_2H_5 HCl	 N—$CO_2C_2H_6$	H	30	294–295	$C_{17}H_{28}N\ O_4Cl$	C	48 71	48 95
						H	6 54	6 76

[a] The melting points were taken on a Fisher Johns block and are uncorrected
[b] The carbon-hydrogen analyses were obtained from the Weiler and Strauss Microanalytical Laboratory, Oxford, England
[c] Measurements of water content were done by determining loss of weight at 100° *in vacuo* (2–5 mm) over phosphorus pentoxide
[d] Determinations of ionizable chloride were done by the Volhard method
[e] The *p* chloroanilino groups in these compounds were reduced to anilino groups

after vacuum drying were also carried out where indicated.

BIOLOGICAL RESULTS

Three of the diamino-*s*-triazines were tested at the *Lilly Research Laboratories for diuretic and blood* glucose effects These compounds, 2,4-bis-(2'-carbethoxyaminoethylamino)-*s*-triazine hydrochloride, 2,4 bis-anilino-*s*-triazine dihydrochloride, and 2,4-bis-(4'-carbethoxypiperazino)-*s*-triazine hydrochloride showed no significant diuresis when tested orally in dogs at dose levels of 5–20 mg /Kg The bis-anilino derivative caused an 18 mg % decrease in blood glucose, lasting less than three hours, at a dose of 250 mg /Kg in rats, p o , and the bis-carbethoxypiperazinyl derivative caused a 14 mg % decrease in blood glucose in seven hours at the same dose level in rats The third *s*-triazine showed no effect on blood glucose at this level.

EXPERIMENTAL

Aminochloro-*s*-triazines.—2,4-Diamino-6-chloro-*s*-triazine was supplied by the American Cyanamid Co 2-Chloro-4,6-bis-(2'-carbethoxyaminoethylamino)-*s*-triazine was prepared by Dr. Lester Chafetz (12d) 2,4-Dichloro-6-(2'-pyridylamino)-*s*-triazine, 2-chloro-4,6-bis-(2'-pyridylamino)-*s*-triazine, 2 4-dichloro-6-(2'-thiazolylamino)-*s*-triazine,

and 2-chloro-4,6-bis-(2′-thiazolylamino)-*s*-triazine were prepared according to Foye and Buckpitt (13). 2 - (4′ - Carbethoxypiperazinyl) - 4,6 - dichloro - *s* - triazine and 2,4-bis-(4′-carbethoxypiperazinyl)-6-chloro-*s*-triazine were prepared according to Foye and Chafetz (12d).

2,4 - Dichloro - 6 - *p* - chloroanilino - *s* - triazine was prepared according to Cuthbertson and Moffatt (14). 2-Chloro-4,6-bis-(*p*-chloroanilino)-*s*-triazine and 2 - amino - 4 - *p* - chloroanilino - 6 - chloro - *s* - triazine were prepared by the general procedure of Thurston (12a). The first of these two compounds was a white powder which melted at 243–245° and was obtained in 92% yield.

Anal.—Calcd. for $C_{15}H_{18}N_5Cl_3$: C, 49.21; H, 2.79. Found: C, 48.87; H, 2.93.

The latter compound, 2-amino-4-*p*-chloroanilino-6-chloro-*s*-triazine, was prepared by forming a slurry of cyanuric chloride (5.0 Gm., 0.027 mole) in 60 ml. of water and 60 ml. of dioxane at 0–5° and adding slowly a solution of *p*-chloroaniline (3.5 Gm., 0.027 mole) in 50 ml. of dioxane. An excess of aqueous sodium bicarbonate was added gradually, and the reaction mixture was stirred at 0–5° for two hours. To this slurry was then added 4.3 ml. (0.027 mole) of ammonia water, and the temperature was raised to 40–50°. The reaction mixture was stirred at this temperature for two hours, and the precipitate was filtered, washed free of chloride ion with ice water, and pressed as dry as possible. The product was dried overnight at 60°. After being washed with hot alcohol and redried overnight at 60°, 5.4 Gm. (78%) of a fine, white powder was obtained which decomposed at 200°.

Anal.—Calcd. for $C_9H_7N_4Cl_2$: C, 42.34; H, 2.77. Found: C, 41.93; H, 2.90.

Amino-*s*-triazines—The following is representative of the reduction procedure. 2,4-Diamino-6-chloro-*s*-triazine (6.0 Gm., 0.04 mole) was dissolved in 200 ml. of propylene glycol (95% ethanol was suitable for the other compounds reduced) with heating. The solution was placed in a three-necked flask fitted with a mechanical stirrer, an inlet tube for hydrogen, and an outlet tube for the hydrogen and other gases not absorbed by the amino-*s*-triazines. Six grams of 10% palladium-on-charcoal (American Platinum Works, Newark, N. J.), dried at 60°, was added to the solution and the temperature of the mixture was maintained at 70–78° during the reaction. Hydrogen was then passed into the stirred mixture for four hours. The evolved gases were bubbled through 0.2 *N* sodium hydroxide solution containing phenolphthalein to detect any hydrogen chloride or hydrogen cyanide liberated. The mixture, while still warm, was filtered to remove the catalyst.

The resulting solution was distilled under reduced pressure to give a residue of 2.7 Gm. (44%) of white 2,4-diamino-*s*-triazine hydrochloride which did not melt below 300°. The product, unlike the starting material, was soluble in alcohol, slightly soluble in water, and gave a positive test for ionizable halogen. Recrystallization of the products was generally successful from alcohols or petroleum ether-alcohol solutions.

SUMMARY

1. Mono- and diamino-*s*-triazines have been prepared in good yield by catalytic hydrogenolysis of halogen in the corresponding cyanuric chloride derivatives using 10 per cent palladium-on-charcoal. The method appears to be general, without causing rupture of the *s*-triazine or other heterocyclic rings, but suffers from the disadvantage that other aromatically-bound halogen is also removed.

2. The following new *s*-triazines were prepared and characterized: 2-chloro-4,6-bis-(*p*-chloroanilino), 2-amino-4-*p*-chloroanilino-6-chloro, 2,4-diamino hydrochloride, 2,4-bis-(2′-carbethoxyaminoethylamino) hydrochloride, 2-anilino hydrochloride, 2,4-dianilino dihydrochloride, 2-amino-4-anilino dihydrochloride, 2-(2′-pyridylamino) dihydrochloride, 2,4-bis-(2′-pyridylamino) hydrochloride, 2-(2′-thiazolylamino) hydrochloride, 2,4-bis-(2′-thiazolylamino) hydrochloride, 2-(4′-carbethoxypiperazinyl) hydrochloride, and 2,4-bis-(4′-carbethoxypiperazinyl) hydrochloride.

3. Three of the diamino-*s*-triazines were tested for oral diuretic activity and showed no significant effects. The bis-anilino and bis-carbethoxypiperazinyl derivatives caused decreases in blood glucose in rats, however.

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Determination of Rotenone in Pharmaceuticals*

By JACK T. BRYAN and WARREN R. LINCOLN

A procedure in which chloroform was used to extract rotenone from a pharmaceutical mixture has been described. A colorimetric assay for rotenone was adapted for use as an analytical tool in product development. Both moisture and heat of reaction were shown to be detrimental in the development of the color for assay.

THE DISCOVERY by Gross and Smith (1) that rotenone will react with sodium nitrite to give a stable red color under certain conditions has served as a basis for the development of a colorimetric assay. The method consisted of treating an acetone solution of rotenone with alcoholic potassium hydroxide and then acidifying with dilute nitric acid containing sodium nitrite. Goodhue (2) reported that this procedure was not entirely satisfactory because of an intense yellow background caused by the reagents. The condition was presumably obviated by Goodhue (2) by substituting sulfuric acid for nitric acid, by diluting the potassium hydroxide with water, and adding sodium nitrite with the alcoholic potassium hydroxide. In 1945, Jones (3) made further modifications to this later procedure in an attempt to obtain increased precision. It was recorded by Jones (3) that purity of the alcohol used to prepare alcoholic potassium hydroxide was important. Alcohol which had been distilled after refluxing with zinc and potassium hydroxide did not give background color. The temperature at which the red color was developed was shown to be of prime importance as well as the manner in which the acid was added to the alkaline solution of rotenone.

The colorimetric procedure for rotenone is not specific since the color can be developed with deguelin, dihydrorotenone, and other related compounds. The analytical methods reported above relate primarily to the assay of rotenone in agriculturally used insecticides. Our immediate concern was the need to adapt this method to the analysis of rotenone, in pure form, in pharmaceutical preparations containing several other active ingredients and vehicle components of the usual emulsion type. These pharmaceuticals are intended to be used in veterinary medicine for the treatment of certain dermatoses. Several modifications were made in the colorimetric determination reported by Jones (3) making it useful for such pharmaceutical products.

* Received March 27, 1959, from the Veterinary Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

EXPERIMENTAL

Materials.—(*a*) Sodium nitrite solution: One gram sodium nitrite was dissolved in 10 cc. of water and diluted to one liter with 95% ethyl alcohol. (*b*) Potassium hydroxide, 40% solution: forty grams potassium hydroxide was dissolved in approximately 80 cc. of water, and after cooling, diluted to 100 cc. with water. (*c*) Alkaline sodium nitrite solution: one volume of 40% potassium hydroxide solution was mixed with 7 volumes of sodium nitrite solution. (*d*) Sulfuric acid, 9 *N:* free from nitrous acid. (*e*) Acetone, U. S. P. (*f*) Chloroform, U. S. P.

Apparatus.—Beckman, Model B, spectrophotometer with 1-cm. cells, water bath adjustable to 28° ± 0.5°.

Procedure.—An accurately measured sample containing approximately 1 mg. of rotenone was transferred to a 60-cc. separatory funnel. Distilled water was added to make a total volume of 5 cc. To this aqueous mixture was added 10 cc. of chloroform and the extraction carried out by shaking for several minutes. After the two phases separated, the chloroformic phase was separated and filtered through a pledget of glass wool into a 100-cc. volumetric flask. The extraction was carried out three times, after which the combined extracts were diluted to 100 cc. Several grams of dried sodium sulfate were added and the flask shaken, then allowed to stand for one hour.

A 10-cc. aliquot of the chloroformic extract was placed in a 25-cc. glass-stoppered Erlenmeyer flask. The chloroform was evaporated on a steam bath after which the flask was placed in a vacuum desiccator containing calcium chloride for a minimum of one hour. After drying, 2 cc. of acetone was added to the residue and the flask swirled gently until complete solution was effected.

A standard was prepared containing 100 μg. of rotenone in 2 cc. of acetone. A blank containing only 2 cc. of acetone was prepared and these were carried through the remaining procedure with the samples.

The flasks containing the sample, standard, and blank were placed in a constant temperature bath at 28° ± 0.5°. At the end of five minutes, 2 cc. of of alkaline sodium nitrite reagent was added and the flasks swirled to insure proper mixing. After reacting for five minutes, the flasks were removed to an ice bath, chilled for approximately one minute, and 5 cc. 9 *N* sulfuric acid was added rapidly from a volumetric pipet. Each flask was swirled vigorously until any solid which precipitated after the addition of the acid was dissolved. The flasks were then placed in the constant temperature water bath at 28° ± 0.5° for fifteen minutes. After the fifteen-minute interval, the color was read in a spectrophotometer at 545 mμ, using the blank to set the instrument. Samples which were cloudy were filtered through No. 5 Whatman filter paper before reading the color.

Calculations.—The mg./ml. of rotenone in the sample was calculated according to the following

formula mg /ml rotenone = $(A_x/A_{st}) \times (St/d)$ where A_x = observed absorbence of sample, A_{st} = observed absorbence of the standard, St = mg of rotenone in standard, and d = dilution of sample

Results.—The establishment of an optimum working range for the sample size was carried out by observing that samples of rotenone from 50 µg to 200 µg follow Beer's law A sample size of 100 µg was found to give a desirable color intensity The stability of developed color with respect to time can be seen in Table I The red color appears to be stable for a period of thirty to sixty minutes at room temperature after development

TABLE I —STABILITY OF COLOR, A_{545}

Time min	S_1	S_2	S_3
10	342	350	350
15	342	352	352
30	339	350	350
60	334	348	347
120	327	338	338

The graph in Fig 1 shows that the length of time for the development of color by the alkaline sodium nitrite is critical and the range of five minutes recorded in the literature (3) is within the range found in our laboratory

Jones (3) has recorded that lowering the temperature of the alkaline nitrite mixture just prior to the addition of sulfuric acid increases the sensitivity of the color reaction However, no investigation of the degree of sensitivity increase was recorded using an ice bath Table II shows that the color intensity of the reaction is significantly increased by chilling for one minute just prior to the addition of sulfuric acid After a cooling period of ten minutes, the color intensity decreases

TABLE II —EFFECT OF CHILLING NITRITE MIXTURE PRIOR TO ADDITION OF SULFURIC ACID, A_{545}

Time min	S_1	S_2	S_3
0 (no chilling)	396	400	
1 (chilling)	470	472	474
10 (chilling)	459	459	461

In an attempt to further eliminate interfering variables, the effect of moisture in the final sample on the color intensity was studied It was found that a greater degree of precision could be obtained between samples if the chloroformic extract containing the rotenone was dried over sodium sulfate before the aliquot for evaporation was removed After the evaporation of chloroform, the flask containing the residue was placed in a vacuum desiccator for one hour Table III shows the results

TABLE III —EFFECT OF MOISTURE IN SAMPLES, A_{545}

No Drying	Variation from Average %	Dried	Variation from Average %
339	+14 1	309	<0 1
264	−11 1	308	<0 1
270	−9 3	311	+0 2
283	−4 7	309	<0 1
330	+11 1	310	<0 1

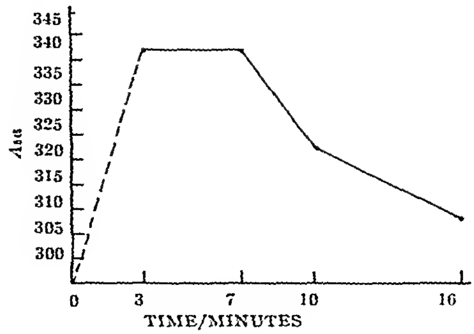

Figure 1

Table IV further substantiates our findings that moisture in the final assay mixture causes a loss of precision and accuracy These samples were assayed, either dry or with moisture present, then increments of rotenone were added and the samples reassayed for total amount of rotenone

TABLE IV —THE EFFECTS OF MOISTURE ON THE DETERMINATION OF ROTENONE

Sample	Found mg /ml	Added mg /ml	Total Found mg	Recovered %
A moist	625	0 50	1 214	117 7
A dried	812	0 50	1 328	103 2
B dried	661	1 00	1 655	99 4
C moist	284	0 50	0 571	57 2
C dried	262	0 50	0 773	101 8
D moist	473	0 50	0 925	90 4
D dried	519	0 50	1 013	98 8

Table V shows the results and standard deviation of a number of extractions and rotenone determinations using the method previously outlined This table also shows that it was quite possible to quantitatively extract rotenone from a typical lotion-type pharmaceutical

TABLE V —DETERMINATION OF ROTENONE IN A PHARMACEUTICAL MIXTURE

Sample	Theory, mg /ml	Found mg /ml	Theory, %
1	1 04	1 04	100
2	1 04	1 02	98
3	1 04	1 00	96
4	1 04	1 05	101
5	1 04	1 03	99
6	1 04	1 06	102
7	1 04	1 02	98
8	1 04	1 03	99
9	1 04	1 03	99
10	1 04	1 02	98
11	1 04	1 02	98
12	1 04	1 01	97
13	1 04	1 02	98
Standard deviation		1 03 ± 0 02	99 ± 2

SUMMARY AND CONCLUSIONS

An extraction procedure in which chloroform removed rotenone and other materials from an emulsion type lotion has been described The

chloroform-soluble ingredients of the emulsion, polyoxyl 40 stearate, glyceryl monostearate, and active constituents, did not appear to interfere with the subsequent color development of the assay. The following conclusions were reached: It was possible to extract rotenone from a typical pharmaceutical mixture of ingredients for external use in veterinary medicine; drying the extracted sample prior to color development produced a more stable and reproducible color reaction; and the use of an ice bath for a short chilling period to dissipate the heat of reaction caused by the addition of sulfuric acid increased the sensitivity of the color reaction.

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The Effect of Mescaline and Yohimbine on the Respiration of Rat Brain Homogenate*

By PAUL SHATZKO and S. K. SIM

By manometric method using conventional Warburg apparatus, the oxygen uptake of rat brain homogenate in glucose, glutamate, and succinate was found to be inhibited by mescaline and yohimbine. Yohimbine inhibited the brain homogenate's respiration in glucose considerably more than it did the respiration in glutamate and in succinate. Mescaline inhibited the homogenate's oxygen uptake in glucose, in glutamate, and in succinate to a similar degree, when the drug and the substrate were added to the brain homogenate at the same time. This is in contrast to the lack of mescaline inhibition of the succinate respiration of chopped brain preparation which was incubated with the drug for two and one-half hours before addition of substrate, as reported by previous investigators.

THE *in vivo* effects in experimental animals and in man of psychotomimetic agents including mescaline and yohimbine have been reported and reviewed in recent years (1, 2, 3). Various investigators have also reported the effect of such agents on the respiratory activity *in vitro* of brain preparations of different animals (3, 4). Quastel and Wheatley (4) found that mescaline and certain other amines inhibited the oxygen uptake of rat brain (chopped whole brain) in glucose, pyruvate, glutamate, and in lactate, but not in sodium succinate. This was later confirmed by Schueler (5) using the same method, that is, by incubating the chopped brain preparation with the drug for two and one-half hours before adding the respiratory substrate in question. Schueler (5) and Stevenson and Sanchez (6) showed the antidotal effect of sodium succinate on mescaline psychosis in humans. Schueler (5) pointed out the rather quick onset of the *in vivo* effect of mescaline in the humans and that it was difficult to correlate it with the *in vitro* results they obtained on the brain preparation.

In the experiments to be reported here, we investigated the effect of mescaline and yohimbine on the oxygen uptake of rat brain homogenate, using glucose, glutamate, and succinate as respiratory substrates, when the substrate and the drug were added to the brain preparation at the same time.

MATERIALS AND METHODS

Wistar albino rats weighing 180–230 Gm. were decapitated, the brain quickly removed, weighed, rinsed in buffer, and homogenized with a motor-operated Teflon pestle in a glass tissue grinder in Krebs-Ringer phosphate buffer, pH 7.3 (7). The tissue grinder and the buffer solution were chilled immediately before use. The final homogenate was made up with the buffer to 10% w/v with respect to the fresh weight of the brain tissue. Oxygen uptake was measured by conventional Warburg apparatus at 38°. Each Warburg reaction vessel contained 1 ml. of the brain homogenate, a substrate, with or without the drug, all dissolved in the same buffer and with a total volume of 3 ml. The center well contained 0.2 ml. of 20% KOH absorbed on filter paper strip. The gas phase was air. The substrate and the drug were tipped in from separate side arms of the reaction vessel at zero time after fifteen-minute equilibration in the constant temperature bath. In each experiment 5–8 vessels contained the homogenate, substrate, and the drug, and a similar number of vessels contained homogenate and substrate without the drug. Manometric readings were taken at first at five-minute and sub-

* Received October 31, 1958, from the research laboratories of the Faculty of Pharmacy, University of British Columbia, Vancouver 8, Canada.
This investigation was supported in part by a grant from the Canadian Foundation for the Advancement of Pharmacy.
Part of this work was presented at the 5th Canadian Conference on Pharmaceutical Research, Edmonton, Alta., August 1958.
The nitrogen determinations were made with the technical assistance of C. Mezei and I. C. Caldwell.

sequently at ten-minute intervals up to ninety minutes.

The substrates used were glucose (0.033 *M*), sodium glutamate (0.2 *M*), and potassium and sodium succinate (0.03 *M*). The drugs used were mescaline hydrochloride (0.03 *M*, 0.09 *M*, and 0.18 *M*), and yohimbine hydrochloride (0.005 *M* and 0.0073 *M*). All concentrations of substrates and drugs refer to the final concentration in the reaction mixture.

Nitrogen content of the homogenate was determined as total nitrogen by a micro-Kjeldahl method modified from those of Ballentine and Gregg (8) and of Pepkowitz and Shive (9).

The rate of oxygen uptake, Q_{O_2}, was based on the rate of the first forty minutes after zero time and calculated as μl. of oxygen per hour per mg. N. In a few cases, as indicated in the results, the rate of oxygen uptake was calculated as μl. of oxygen per hour per ml. of homogenate.

RESULTS

The oxygen uptake figures, at different time intervals, by 1 ml. of the brain homogenate in the presence of the substrate with and without the drug are plotted in Figs. 1, 2, and 3. Each oxygen uptake figure, in μl., is the mean of figures obtained from 5–18 reaction vessels in several typical experiments. Table I lists the rates of oxygen uptake as μl. of oxygen per hour per mg. of nitrogen except for those otherwise indicated as being μl. of oxygen per hour per ml. of homogenate. Although the brain homogenates used in different experiments on different days had different nitrogen contents ranging from 1.4 to 2.3 mg. N per ml. of homogenate, comparisons of the rates of oxygen uptake in the presence of any particular substrate with the drug and without the drug were made on the same batch of homogenate used on the same day. Therefore, the percentage of inhibition of rate of oxygen uptake by the drug would be the same whether this is calculated by

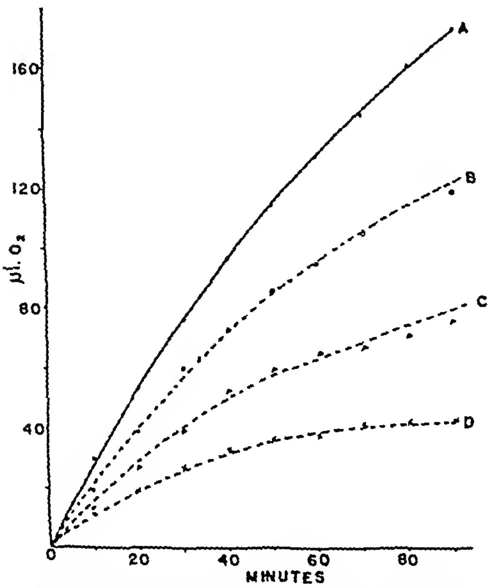

Fig. 1.—The effect of different concentrations of mescaline on oxygen uptake of rat brain homogenate in glutamate. A—glutamate (0.2 *M*); B, C, D—glutamate 0.2 *M* with mescaline 0.03 *M*, 0.09 *M*, and 0.18 *M*, respectively.

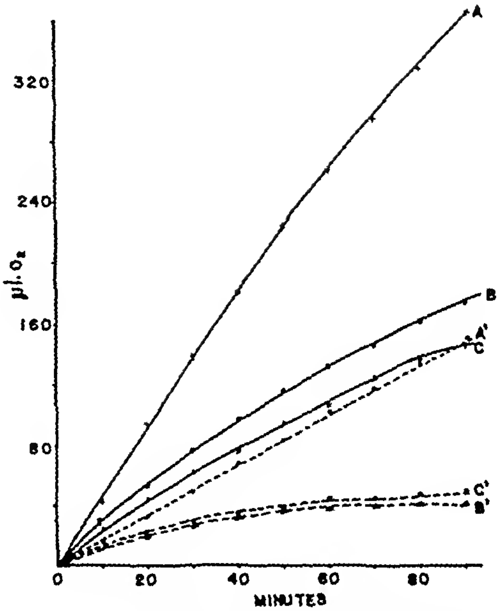

Fig. 2.—The effect of mescaline on oxygen uptake of rat brain homogenate in succinate, glutamate, and glucose. A—potassium succinate (0.03 *M*); A′—succinate + mescaline (0.18 *M*); B—glutamate (0.2 *M*); B′—glutamate + mescaline (0.18 *M*); C—glucose (0.033 *M*); C′—glucose + mescaline (0.09 *M*).

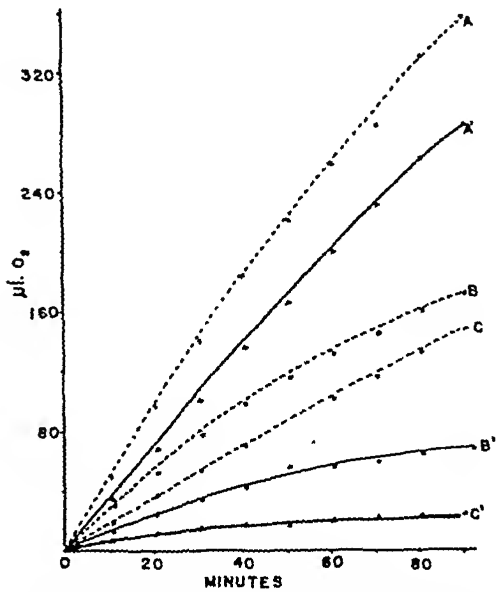

Fig. 3.—The effect of yohimbine on oxygen uptake of rat brain homogenate in various substrates. A—succinate (0.03 *M*); A′—succinate + yohimbine (0.0073 *M*); B—glutamate (0.2 *M*); B′—glutamate, + yohimbine (0.0073 *M*); C—glucose (0.033 *M*); C′—glucose + yohimbine (0.0073 *M*).

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In the experiments to be reported here, we investigated the effect of mescaline and yohimbine on the oxygen uptake of rat brain homogenate, using glucose, glutamate, and succinate as respiratory substrates, when the substrate and the drug were added to the brain preparation at the same time.

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sequently at ten minute intervals up to ninety minutes

The substrates used were glucose (0 033 M), sodium glutamate (0 2 M), and potassium and sodium succinate (0 03 M) The drugs used were mescaline hydrochloride (0 03 M, 0 09 M, and 0 18 M), and yohimbine hydrochloride (0 005 M and 0 0073 M) All concentrations of substrates and drugs refer to the final concentration in the reaction mixture

Nitrogen content of the homogenate was determined as total nitrogen by a micro Kjeldahl method modified from those of Ballentine and Gregg (8) and of Pepkowitz and Shive (9)

The rate of oxygen uptake, Q_{O_2}, was based on the rate of the first forty minutes after zero time and calculated as µl of oxygen per hour per mg N In a few cases, as indicated in the results, the rate of oxygen uptake was calculated as µl of oxygen per hour per ml of homogenate

RESULTS

The oxygen uptake figures, at different time intervals, by 1 ml of the brain homogenate in the presence of the substrate with and without the drug are plotted in Figs 1, 2, and 3 Each oxygen uptake figure, in µl, is the mean of figures obtained from 5-18 reaction vessels in several typical experiments Table I lists the rates of oxygen uptake as µl of oxygen per hour per mg of nitrogen except for those otherwise indicated as being µl of oxygen per hour per ml of homogenate Although the brain homogenates used in different experiments on different days had different nitrogen contents ranging from 1 4 to 2 3 mg N per ml of homogenate, comparisons of the rates of oxygen uptake in the presence of any particular substrate with the drug and without the drug were made on the same batch of homogenate used on the same day Therefore, the percentage of inhibition of rate of oxygen uptake by the drug would be the same whether this is calculated by

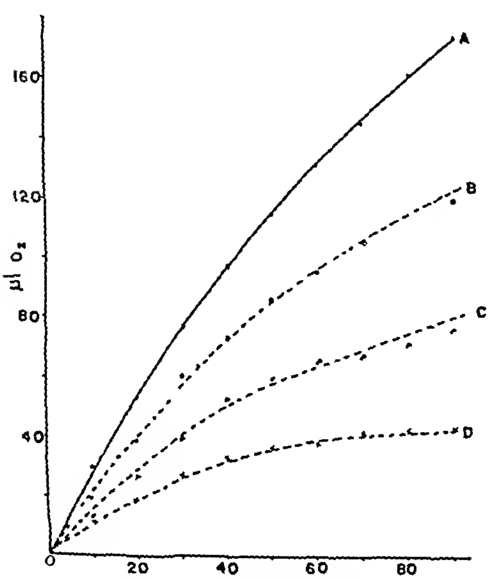

Fig 1—The effect of different concentrations of mescaline on oxygen uptake of rat brain homogenate in glutamate A—glutamate (0 2 M), B, C, D—glutamate 0 2 M with mescaline 0 03 M, 0 09 M, and 0 18 M, respectively

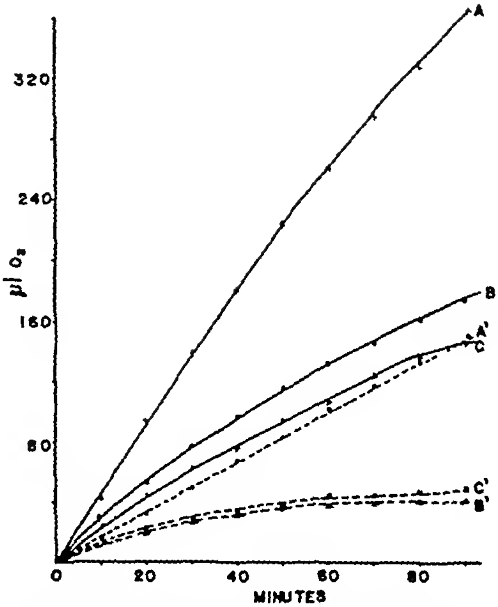

Fig 2—The effect of mescaline on oxygen uptake of rat brain homogenate in succinate, glutamate, and glucose A—potassium succinate (0 03 M), A'—succinate + mescaline (0 18 M), B—glutamate (0 2 M), B'—glutamate + mescaline (0 18 M), C—glucose (0 033 M), C'—glucose + mescaline (0 09 M)

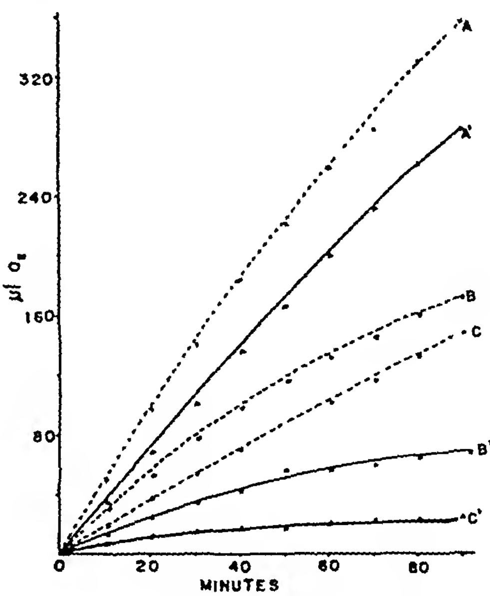

Fig 3—The effect of yohimbine on oxygen uptake of rat brain homogenate in various substrates A—succinate (0 03 M), A'—succinate + yohimbine (0 0073 M), B—glutamate (0 2 M), B'—glutamate, + yohimbine (0 0073 M), C—glucose (0 033 M), C'—glucose + yohimbine (0 0073 M)

TABLE I —THE EFFECT OF MESCALINE AND YOHIMBINE ON THE OXYGEN UPTAKE OF RAT BRAIN HOMOGENATE

Expt No	Substrate and Concentration	Q_{O_2} in Presence of Substrate µl /hr / mg N	Q_{O_2} in Presence of Substrate plus Drug % Inhibition in Brackets Mescaline 0 03 M	Mescaline 0 09 M	Mescaline 0 18 M	Yohimbine 0 005 M	Yohimbine 0 0073 M
12	Glucose	107 1[a]	67 0[a] (37 4%)				
28	0 033	103 1[a]		37 1[a] (64 1%)			
26	M	40 2					6 7 (83 2%)
32		62 0					11 0 (82 3%)
13	Sodium	132 2[a]	103 0[a] (21 9%)				
14	gluta	134 9[a]		77 9[a] (42 2%)			
22	mate	65 1			22 6 (65 2%)		
39	0 2 M	89 8[a]			37 7[a] (58%)		
16		53 7				36 5 (32%)	
18		75 1					39 5 (47 4%)
19		76 8					37 8 (50 8%)
20	Potassium	139 0		79 8 (42 5%)			
21	succin	128 0		81 8 (36 1%)			
24	ate 0 03	120 9			49 5 (59%)		
23	M	129 6				113 2 (12 6%)	
25		115 8					92 2 (20 3%)
27		130 2					103 6 (20 4%)
36		211 2					169 9 (19 6%)
37	Sodium	87 4		48 7 (44 3%)			
38	succin ate 0 03 M	82 6		44 9 (46 1%)			

[a] Calculated as µl /hr /ml of homogenate

comparing the rates as µl of O per hour per mg N or by comparing the rates as µl of O_2 per hour per ml of homogenate

It will be seen from Table I that both yohimbine and mescaline, at the concentrations used, inhibited the oxygen uptake of the brain homogenate respiring in glucose or glutamate or succinate Yohimbine at 0 0073 M inhibited the respiration in glucose (82%) to a greater degree than it did the respiration in glutamate (50%) and in succinate (20%) The inhibition by mescaline of the respiration in the three substrates was less varied than in the case of yohimbine At 0 09 M mescaline inhibited the respiration in glucose, glutamate, and succinate by 64%, 42%, and 42%, respectively There was no significant difference in the inhibition of oxygen uptake by mescaline when using potassium succinate or sodium succinate as the substrate As mentioned earlier, Quastel and Wheatley (4) and Schueler (5) found that mescaline inhibited the oxygen uptake of their rat brain preparation in glucose and in glutamate but not in sodium succinate In their experiments, the brain preparation was of chopped whole brain and it was incubated with the drug for two and one half hours before addition of the respiratory substrate Our present results, with the use of brain homogenate, and with addition of mescaline and the substrate at the same time, show that mescaline inhibits the oxygen uptake in succinate as well as in glucose and in glutamate under these conditions While one cannot readily correlate such *in vitro* experimental data with sodium succinate's antidotal effect on mescaline psychosis in humans, further studies on the effects of mescaline and other psychotomimetic agents on the respiratory activity of brain tissues would seem to be warranted as attempts toward elucidation of the mechanism of action of these agents

SUMMARY

1 A study of the effects of mescaline and yohimbine on the oxygen uptake of rat brain homogenate with glucose, glutamate, and succinate as respiratory substrates has been made

2 Mescaline at 0 09 M concentration was found to inhibit the brain homogenate's respiration in glucose, in glutamate, and in succinate by 64%, 42%, and 42%, respectively At 0 18 M, mescaline inhibited the respiration in glutamate and in succinate by 65% and 59%, respectively

3 The brain homogenate's respiration in glucose, in glutamate, and in succinate was inhibited by 0 0073 M yohimbine by 82%, 47%, and 20%, respectively

4 The extent of mescaline's inhibition of the brain homogenate's respiration in succinate was essentially the same, whether potassium or sodium succinate was used

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Diuretic Activity of Various Compounds as Determined by Urinary Excretion Studies in Rats*

By AMOS E. LIGHT

In the procedure described for evaluating diuretic activity in rats preliminary forced hydration of the animals may be omitted before the administration of the test dose which in itself does produce some hydration. The rats were starved overnight and the dose given orally or intraperitoneally in 0.5 ml. of water for each 100 Gm. weight. Three animals, each weighing 150–250 Gm., were dosed and grouped together in a metabolism cage resting on a polyethylene funnel for urine collection at four-hour and twenty-hour intervals. Values for urine volume, pH, sodium, and chloride were recorded. Ranges for the usual responses following water administration alone have been defined statistically for control values and amounts above these indicated typical diuretic responses. These actions were divided into two categories: natriuretic activity which increased sodium excretion in a large volume with high pH; and saluretic activity which may be further differentiated into: (*a*) pyrimidine, purine, and chlorothiazide types—immediate volume increase with saluresis; (*b*) mercurials—variable increased volume and sodium but decreased chloride excretion in twenty-hour period; and (*c*) miscellaneous types—saluretic activity in limited dose ranges such as with some triazines. Dose-response curves were obtained with known diuretics, and combinations were studied.

A DIURETIC has been defined as an agent that stimulates the secretion of urine; but since urine is composed of water, inorganic salts, and various organic compounds, all in changing amounts, this description appears to be rather ambiguous, especially when present-day interpretations are considered (1). Several different methods for evaluating diuretics in rats have been proposed (2–6). Many investigators have attempted to simulate the edema found in human pathology by forcing extra fluid into the animals, but this added treatment has been questioned (5). In the present study, certain specific excretory effects of accepted diuretics have been compared in both forcibly hydrated and in untreated animals. These included urine volume, pH, sodium, and chloride values with relation to the time of administration of the drugs. Other diuretics, individually and in combinations, have also been investigated in nonhydrated animals. Several new chemical derivatives were likewise tested for diuretic activity. Sex hormones and sulfhydryl binding compounds were also observed for effects on diuresis.

METHODS

In order to determine the types of diuretic responses suggested above, each dose of a compound under investigation was tested in groups of three male rats, each animal weighing 150–250 Gm. A single group was kept in a wire cage, 9 inches in diameter, placed over an 11-inch polyethylene funnel. A 3/16-inch mesh stainless steel wire screen under the cage prevented fecal material from entering the beaker used to collect the urine sample. Water intake of the animals was determined by marking the water bottles at the conclusion of the various time periods. Spillage of drinking water into the urine sample was prevented by the introduction of a stainless steel trough under the regular water pan. This conducted any overflow away from the funnel opening and precluded any question as to the source of the urine volume especially when the compounds under test stimulated increased consumption of water.

Food was removed from the animals some twenty hours before the test, but water was always available unless noted. The drugs were usually given as solutions or suspensions in 0.5 ml. of water per 100 Gm. rat. Extra hydration was accomplished for certain comparative tests by the oral administration of 2.5 ml. water per 100 Gm. weight one hour before dosing and then 5.0 ml. per 100 Gm. weight at the time of dosing. During the remainder of the test period these forcibly hydrated animals did not have water available in the cage.

The urine volumes were measured for the periods indicated and the pH values determined in a pH meter[1] before appropriate dilutions, necessary for sodium and chloride quantitation. Sodium was determined in a flame photometer,[2] and chloride by the Volhard-Harvey technique (7).

In the diuretic screening tests of new compounds the fiducial limits of values obtained from control groups given water only, 0.5 ml. per 100 Gm. rat, were calculated; and the doses that produced values greater than these amounts were considered as having certain diuretic activities. These were compared with results from the known diuretics. Usually the excretory values of the four-hour and the following twenty-hour periods were obtained

RESULTS AND DISCUSSION

As may be seen in Fig. 1, forced hydration along with acetazolamide[3] given intraperitoneally pro-

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The author wishes to thank Mr Gerd Borner for his technical assistance

[1] pH Meter, Model G, Beckman Instrument Co, South Pasadena, Calif
[2] Flame Photometer, Process and Instruments, 15 Stone Ave, Brooklyn, N Y.
[3] Diamox—Lederle Laboratories Div, American Cyanamid Co, Pearl River, N. Y.

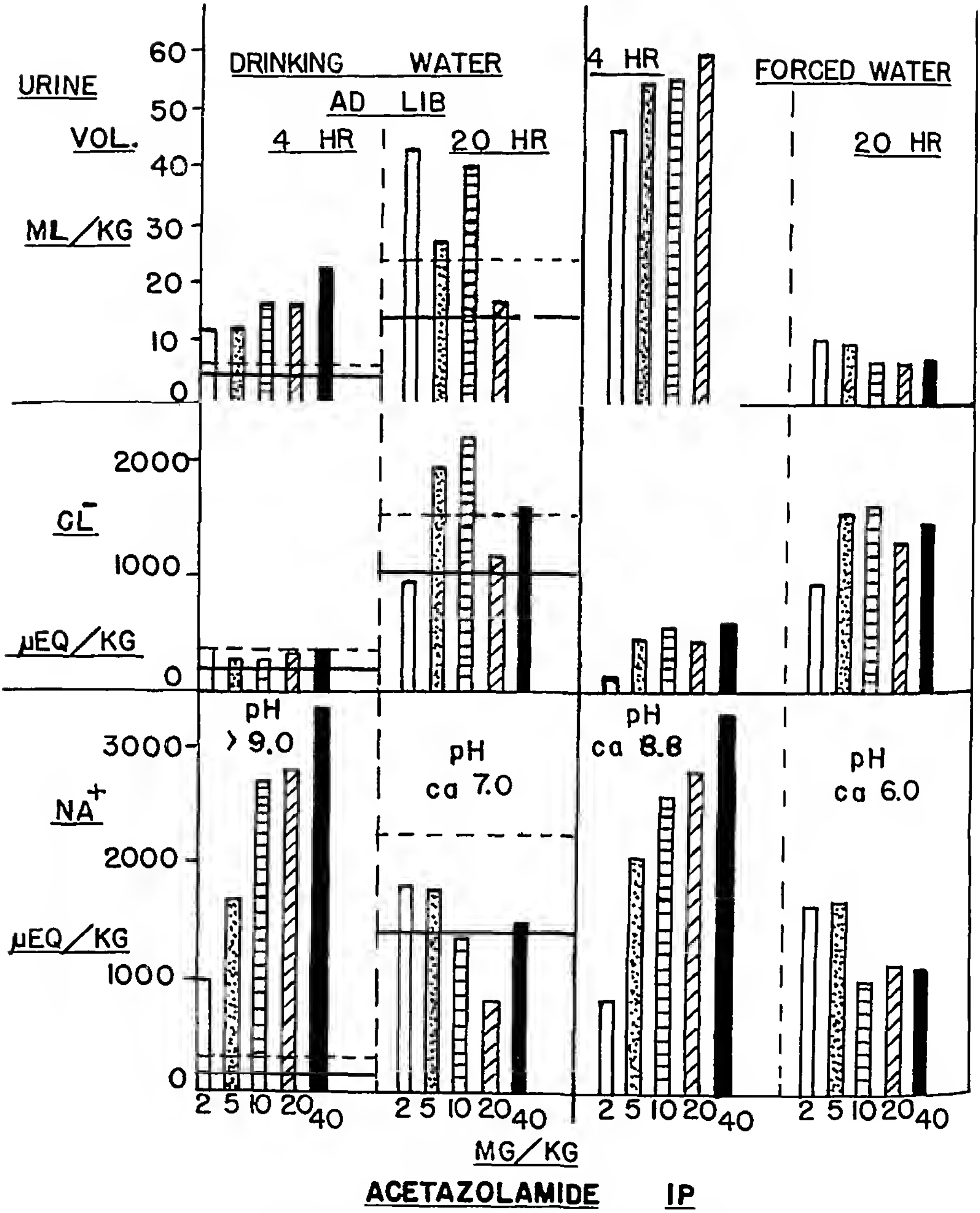


Fig. 1.—Urinary outputs produced by acetazolamide given intraperitoneally to rats with and without preliminary hydration. Heavy horizontal lines in first two sections represent average control values, and the broken lines above each are the fiducial limits (2 × S. D.). The pH values are recorded above the sodium bar graphs.

duced more urine volume at all dose levels than with the water given *ad libitum* during the first four hours. However, the actual increases in volumes from the lower doses to the higher ones were about the same in both procedures. In fact, the per cent increases were obviously much greater in the minimally treated animals. No significant difference was found between the procedures in comparable sodium, chloride, and pH values during the four-hour period. In both, pronounced natriuresis was exhibited with values of over 3,000 microequivalents of sodium per Kg. for this period. With the higher doses the pH likewise increased to 9 from a control range of 6–7. The chloride excreted, however, did not rise above the values usually found in the control group. These control values are summarized in Table I for convenience. The volume output for the forcibly hydrated animals was low during the twenty-hour period for these rats received no drinking water. On the other hand, the animals

TABLE I —URINE VALUES AND WATER INTAKE OF CONTROL RATS

	4 Hrs	Std Dev	20 Hrs	Std Dev	24 Hrs	Std Dev
Volume, ml /Kg	3 47	1 30	13 71	5 01	16 78	5 18
Na^+, micro-equiv /Kg	149 00	81 00	1,358 00	435 00	1,501 00	351 00
Cl^-, micro-equiv /Kg	185 00	78 00	1,049 00	234 00	1,214 00	244 00
pH	6 24	0 28	6 48	0 30		
Water intake, ml /Kg			35 8	13 6		

Drinking water *ad libitum* and water dose given intraperitoneally, 0 5 ml per 100 Gm rat

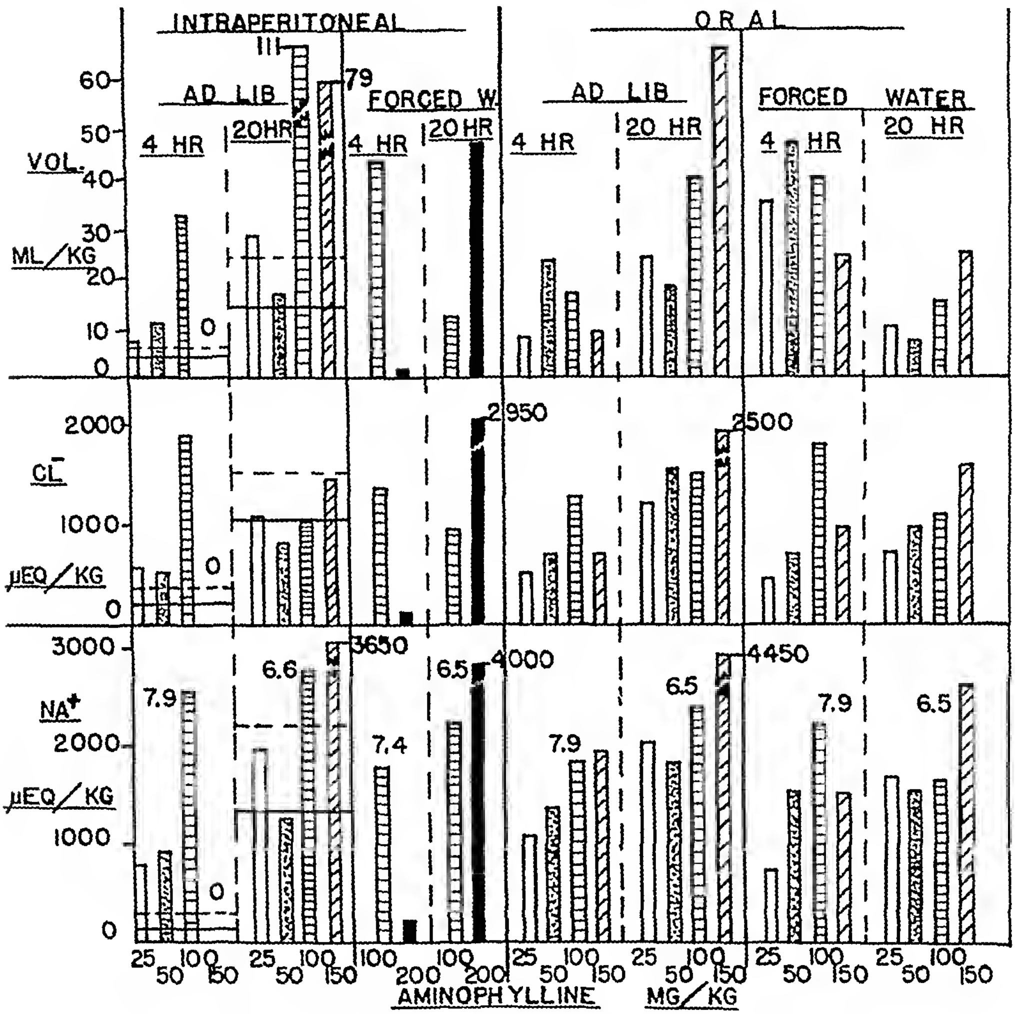


Fig 2 —Comparison of results obtained with aminophylline given orally and intraperitoneally, with and without hydration of animals.

given water *ad libitum* yielded somewhat larger amounts of chloride and volume Both methods gave indications that sodium was possibly being retained to some extent to balance the high excretion rate of the first four hours

The forced hydration method was further studied with aminophylline which was given orally or intraperitoneally. As shown in Fig. 2, the excretion values increased with increasing dosage until a maximum was reached and then decreased to zero in the four-hour period with the dose of 150–200 mg given intraperitoneally. The high doses actually inhibited urine production in the four-hour period. The saluretic action was pronounced in the following tweny hours After oral administration the limiting effect of the high doses was not marked in the four-hour period because the drug was possibly absorbed more slowly The rats given water *ad libitum* had more saluretic action in the twenty-hour period than those forcibly hydrated In all cases the pH never exceeded 8 even at the highest doses in the four-hour period, and the twenty-hour period values were all within the control range Aminometradine[4] gave a similar set of results (Fig 3); however, the saluretic action was

[4] Mictine—G D Searle and Co , Chicago, Ill

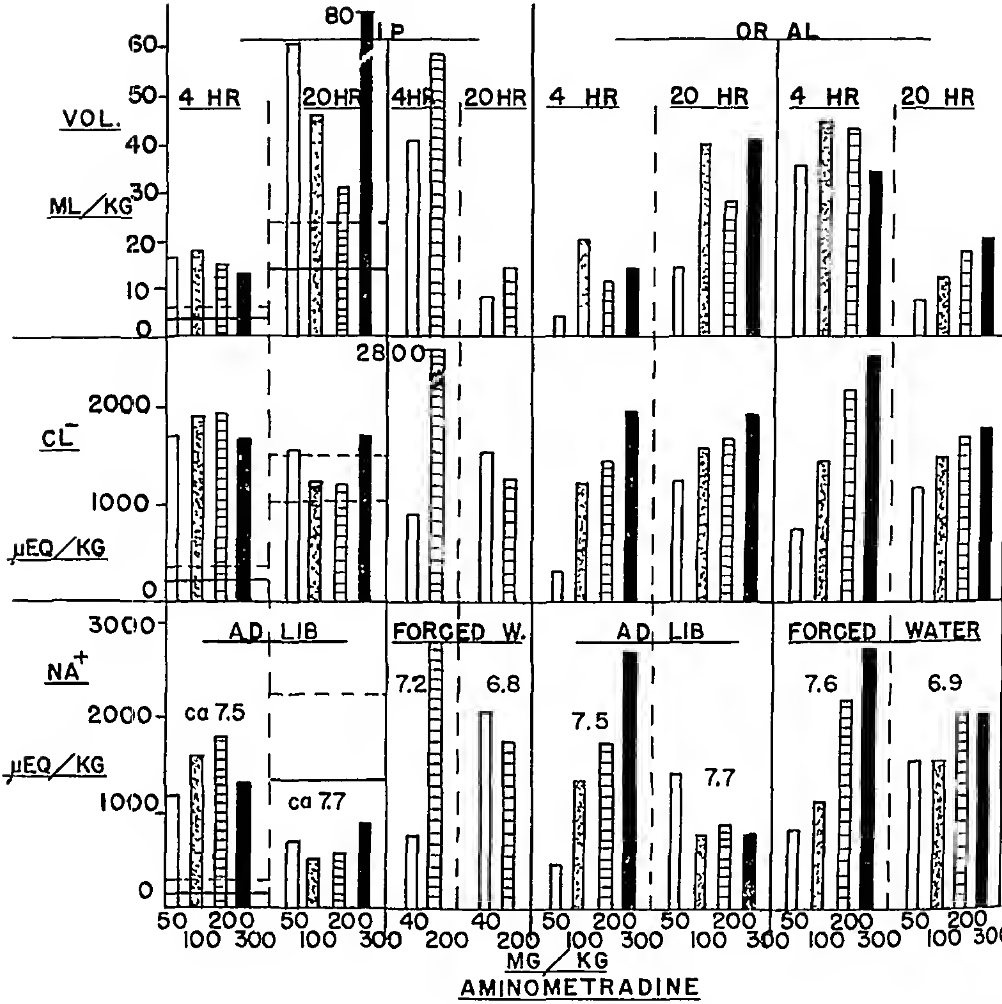


Fig 3—Comparison of results obtained with aminometradine given orally and intraperitoneally with and without hydration

more apparent in the early four-hour period, although the pH values did not rise so much In this one instance forced hydration did appear to increase the sodium output in the twenty-hour interval Other purines studied included theophylline, xanthine, caffeine, and theobromine These were given intraperitoneally with water *ad libitum* As shown in Fig 4, theophylline and caffeine exhibited saluretic action at the doses employed during the four-hour period with emphasis on volume secretion during the twenty-hour period after caffeine administration This latter result was probably due to a thirst factor which caused the rats to drink more water When water was withheld, very little urine was produced In the four hour period after theophylline the pH of the urine rose to not over 7 58 while in the case of caffeine this figure never exceeded 6 87 All other values were within the control range Xanthine and theobromine had no activities at doses of 10 mg or 100 mg per Kg weight

Chlorothiazide[5] given intraperitoneally produced marked saluretic action during the immediate four-hour period after dosing with or without drinking water being available, see Fig 5 Aminoisometradine[6] gave similar but possibly less striking results However, more volume excretion was apparent in the twenty-hour period, thought to be due to the thirst factor stimulated by this drug The pH values following chlorothiazide were around 8 after four hours, whereas after aminoisometradine they reached only 6 9

With mercurial diuretics the dose level of mercury appears to be most critical for obtaining diuresis in the rat (5) Less than 2 mg of mercury per Kg rat gives little indication of diuretic activity, whereas amounts over 8 mg per Kg suppress urine flow At levels of 16 mg and 32 mg definite damage in the rat kidney has been demonstrated histologically (8, 9) In the present study the action of chlormerodrin,[7] a mercurial urea derivative

[5] Diuril—Merck Sharp and Dohme Philadelphia Pa

[6] Rolicton—G D Searle and Co Chicago Ill
[7] Neohydrin—Lakeside Laboratories Inc, Milwaukee Wis

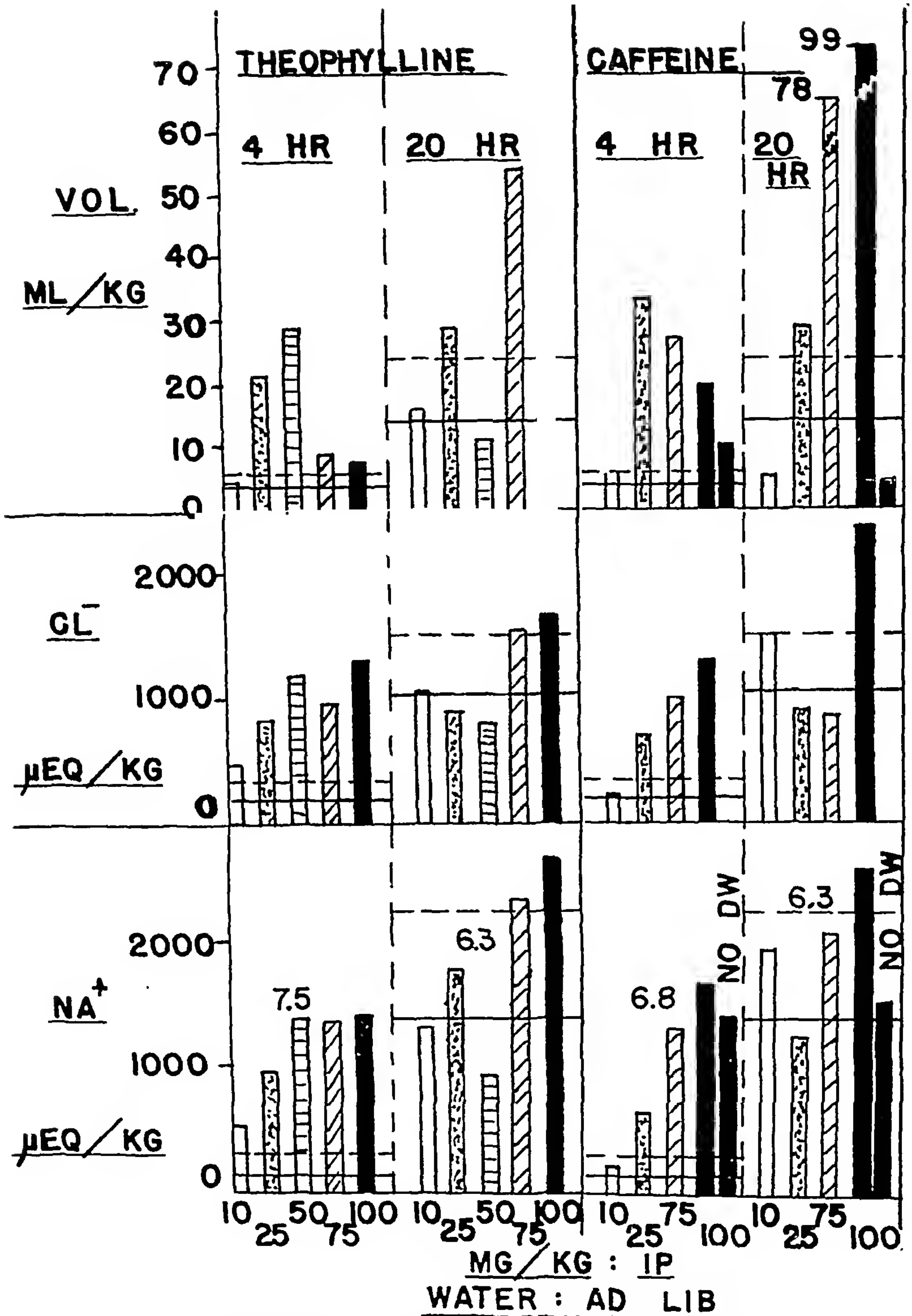
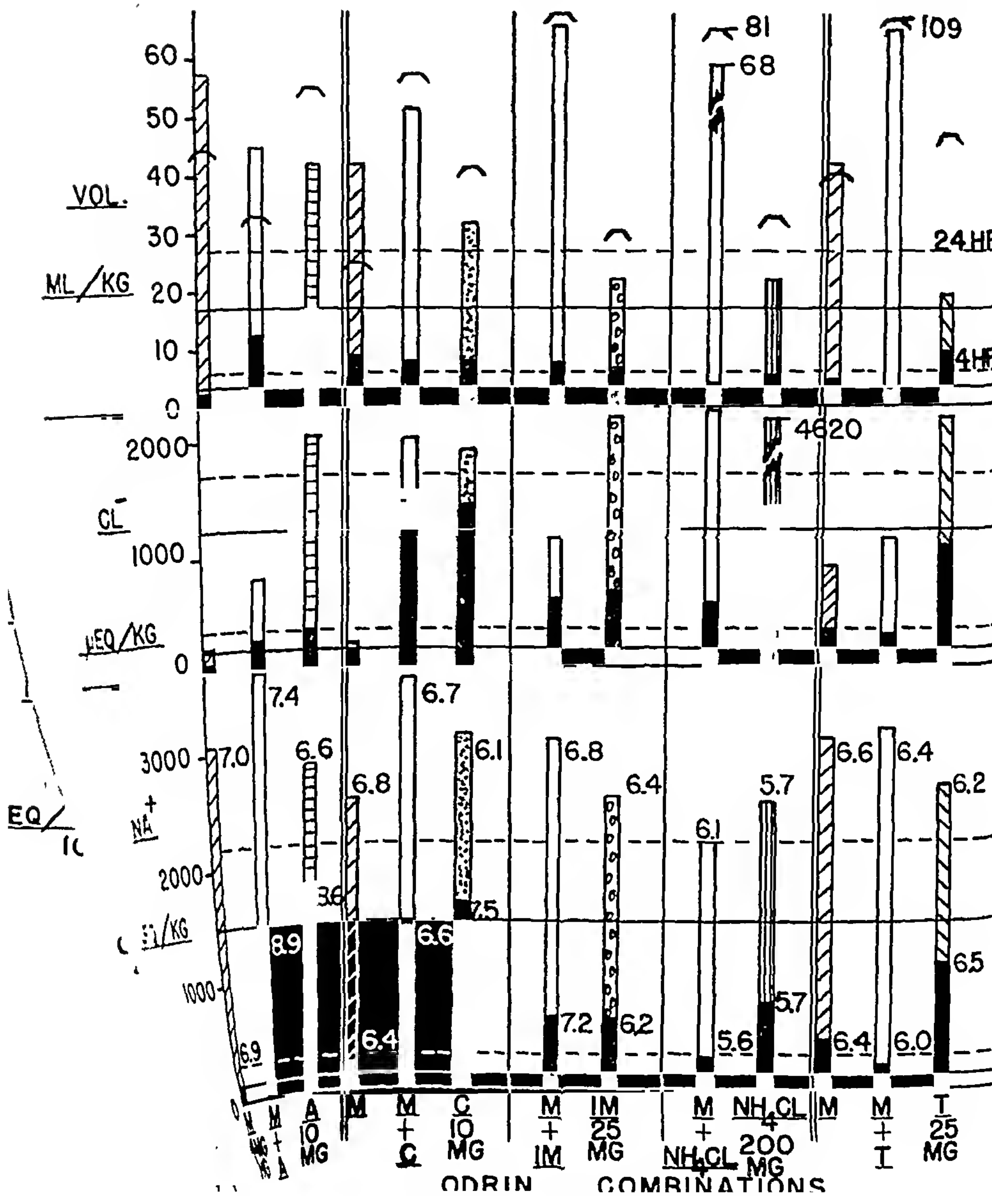


Fig. 4.—Urinary outputs produced by theophylline and caffeine given intraperitoneally to animals receiving water *ad libitum*. (No DW = no drinking water available.)



VOL.
ML/KG
60
50
40
30
20
10
0
81
68
109
24 HR
4 HR
CL⁻
μEQ/KG
2000
1000
0
4620
NA⁺
3000
2000
1000
7.4
7.0
6.6
6.8
6.7
6.1
6.8
6.4
5.7
6.1
6.6
6.4
6.2
3.6
8.9
7.5
6.6
6.4
6.9
7.2
6.2
5.6
5.7
6.4
6.0
6.5
M
A 10 MG
M + C
C 10 MG
M + IM
IM 25 MG
M + NH₄CL
NH₄CL 200 MG
M + T
T 25 MG
ODRIN COMBINATIONS

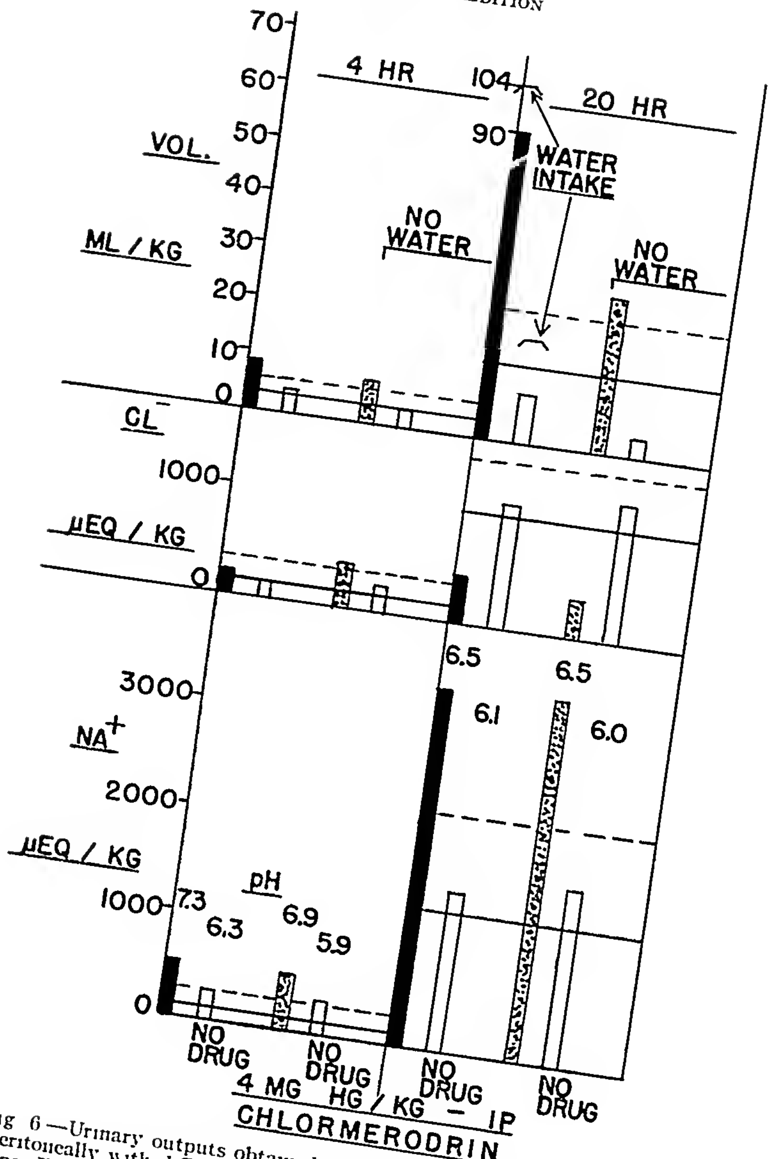


Fig 6—Urinary outputs obtained from chlormerodrin given intraperitoneally with different amounts of water at a dose level of 4 mg per Kg of mercury

metradine the twenty-hour volume was enhanced along with water intake Chloride amounts also rose, and the pH in the four-hour period was slightly elevated With 8 chlorotheophylline the water intake, urine output, and sodium excretion were all enhanced in the twenty-hour period However, the pH values did not change to any extent

Combinations of nonmercurial diuretics with aminoisometradine are illustrated in Fig 8 The addition of acetazolamide enhanced the sodium output and pH values but not necessarily volume which was magnified with chlorothiazide The urine chloride was definitely increased with 8 chlorotheophylline. The latter compound also stimulated the chloride excretion when added to chlorothiazide Acetazolamide and chlorothiazide together increased the four-hour sodium output along with higher pH values similar to the action of the carbonic anhydrase inhibitor alone

Results from different dose levels and combinations of acetazolamide and 8 chlorotheophylline are shown in Fig 9 At low dose levels these appeared to potentiate each other in liberating sodium in the four hour period Other experiments not recorded here with 5 mg per Kg doses of acetazolamide indicated that added caffeine, 25 mg per Kg, had no appreciable effect on the usual result Added triamcinolone[5] 0 5 mg per Kg, appeared to increase sodium excretion to a slight extent Such synthetic corticosteroids have been reported to be antagonists of aldosterone, a possible antidiuretic

[5] Aristocort—Lederle Laboratories Div, American Cyanamid Co, Pearl River N Y

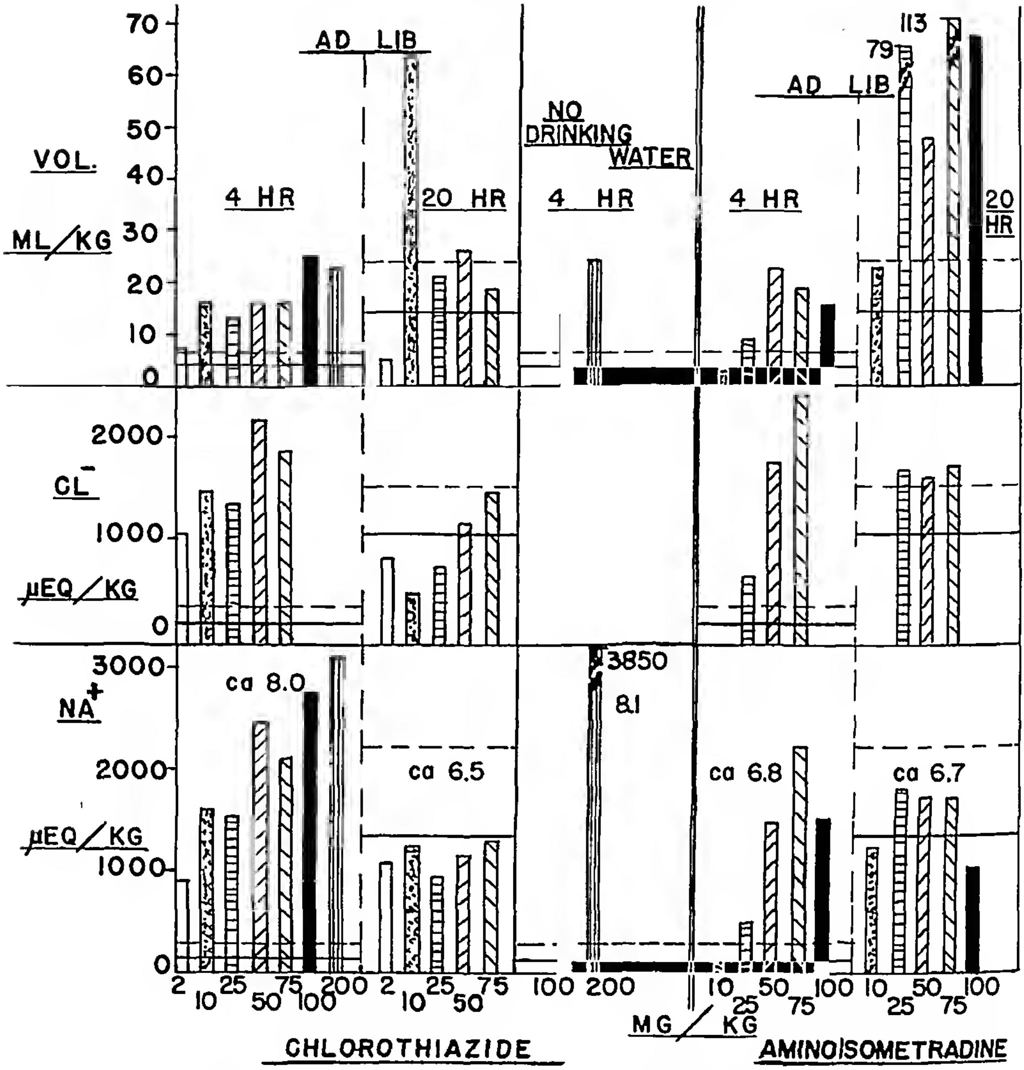


Fig. 5.—Urinary outputs obtained from chlorothiazide (with and without drinking water) and aminoisometradine given intraperitoneally to animals receiving drinking water *ad libitum.*

has been investigated. Doses containing 4 mg. of mercury per Kg. weight were injected intraperitoneally in animals which were with and without drinking water *ad libitum.* As shown in Fig. 6 there was some increase in urine volume and saluretic action in the four-hour period under both conditions. The pH values of the urine from the drug treated animals were likewise higher than their controls, 7.3 and 6.9 as compared to 6.3 and 5.9, respectively. In the twenty-hour period the mercury salt appeared to stimulate thirst when drinking water was available, with the resultant increase in urine flow. However, even when drinking water was not present the production of urine was considerably greater than that of the control group. In both cases sodium excretion was increased along with slightly higher pH values. On the other hand the chloride output seemed to be depressed.

The same dose of this mercurial given in volumes of 0.1 ml. or 2.5 ml. per 100 Gm. weight instead of the usual 0.5 ml. volume gave similar values except that in the four-hour period only, the urine volumes were somewhat less with the lower volume and slightly greater with the larger amount, as would be expected.

It is interesting to note that even with ammonium chloride the mercury salt tended to suppress chloride output as shown in Fig. 7. The acidic salt kept the pH of the urine at 6 or below. This combination caused both an increased consumption of water and a pronounced volume of urine. Other combinations with the mercury salt likewise gave interesting results. With acetazolamide the chloride output was minimized, but sodium excretion was intensified. Water intake remained below the urine volume output. The pH was elevated in both the four-hour and twenty-hour periods. On the other hand, chlorothiazide increased chloride values along with the sodium but did not change the pH to any marked degree. With aminoiso-

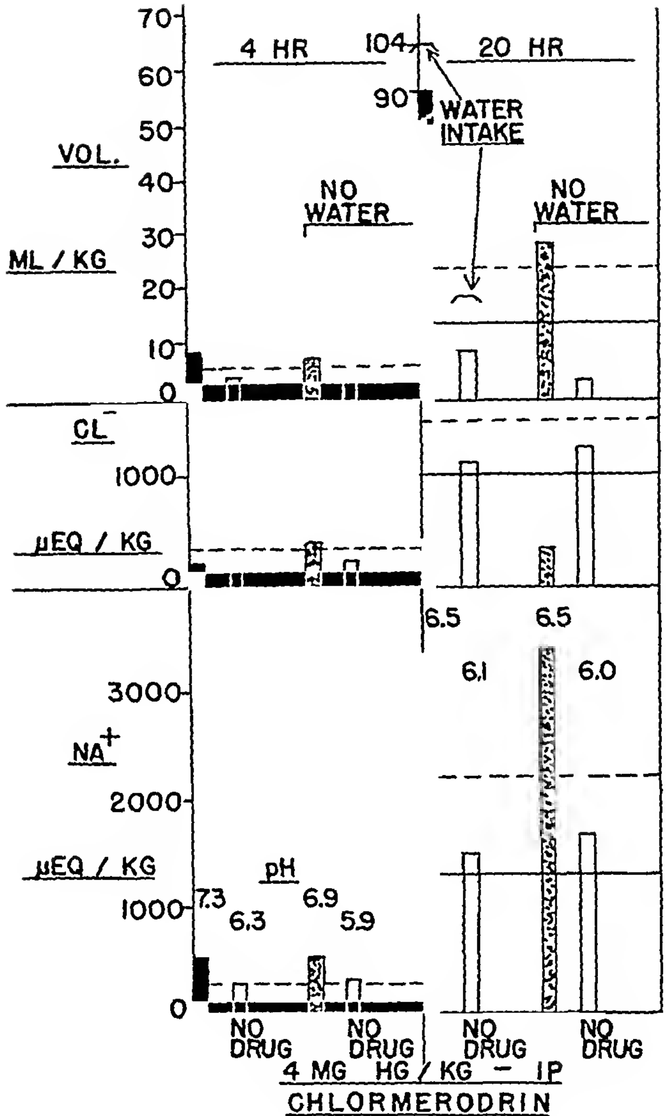


Fig 6—Urinary outputs obtained from chlormerodrin given intraperitoneally with different amounts of water at a dose level of 4 mg per Kg of mercury

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[5] Aristocort—Lederle Laboratories Div American Cyanamid Co Pearl River N Y

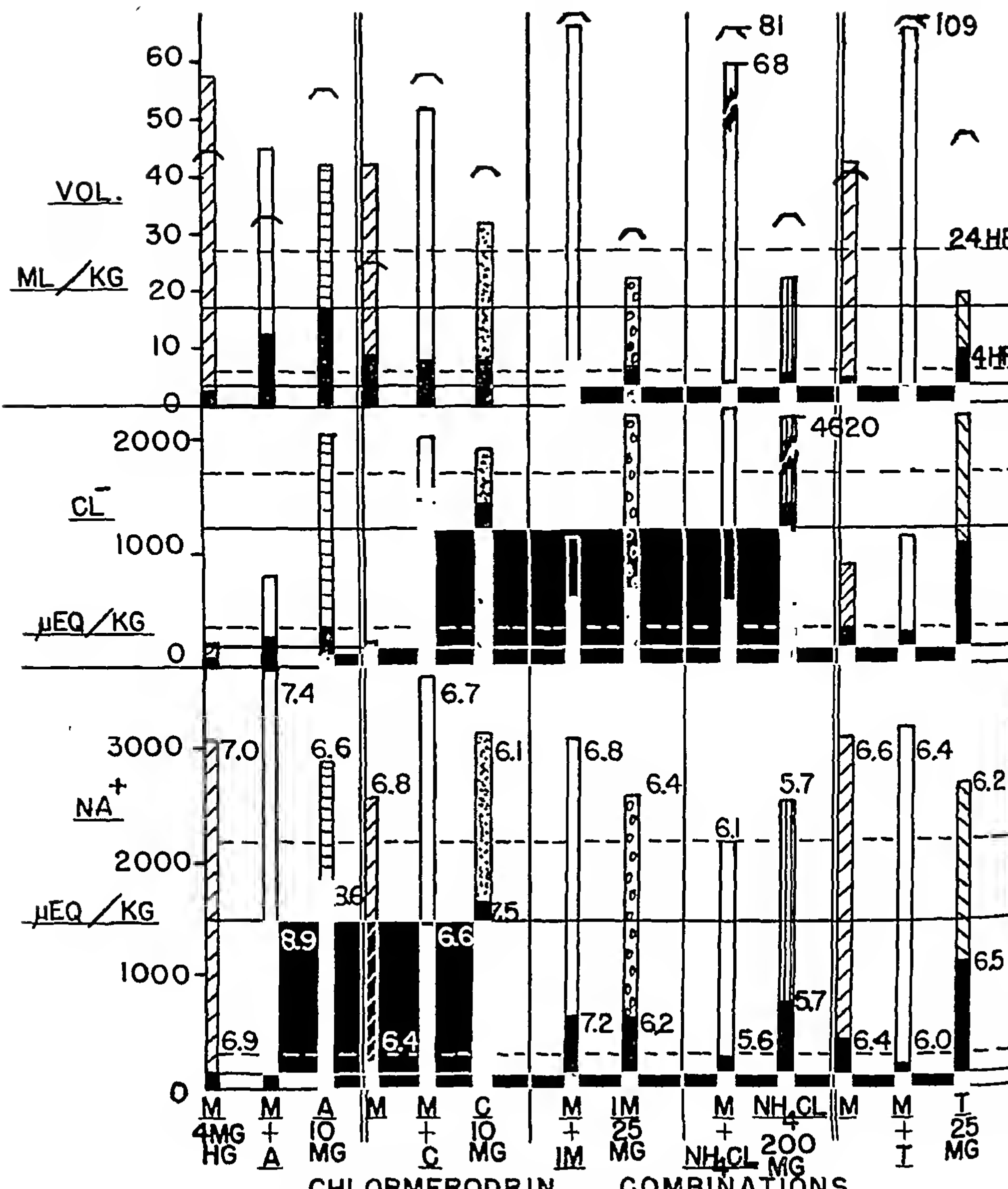

Fig. 7.—Results produced by combinations of chlormerodrin, 4 mg. of mercury per Kg. weight, with non-mercurial diuretics given intraperitoneally to rats receiving drinking water *ad libitum*. Horizontal brackets indicate volume of water consumed in the twenty-hour period. The lower two horizontal lines, solid and broken, in each section are averages for control values and upper fiducial limit (2 × S. D.) for four-hour period; the upper pair are for the twenty-hour period. M (chlormerodrin) + A (acetazolamide), 10 mg. per Kg. M (chlormerodrin) + C (chlorothiazide), 10 mg. per Kg. M (chlormerodrin) + IM (amino-isometradine), 25 mg. per Kg. M (chlormerodrin) + NH_4Cl, 200 mg. per Kg. M (chlormerodrin) + T (8-chlorotheophylline), 25 mg. per Kg.

hormone (10). Theophylline, 25 mg. per Kg., with acetazolamide increased sodium along with volume output and water consumption. Ammonium chloride, 200 mg. per Kg., intensified the chloride output and slightly lowered the pH values, as would be expected.

Various compounds have been screened for diuretic activity by the above procedure. These included purines, pyrimidines, pyrrolopyrimidines, triazines, and other miscellaneous types. Examples are given in Table II. Due to either toxicity or inactivity at low dosages none of these were considered useful diuretics in themselves. However, the activity of low doses of acetazolamide, 5 mg. or 10 mg. per Kg., was markedly enhanced by the co-administration of 10 mg. or 20 mg. per Kg. of 4-(sec-butylamino)-pyrrolo-[2,3-δ]-pyrimidine (B.W. 57-10).

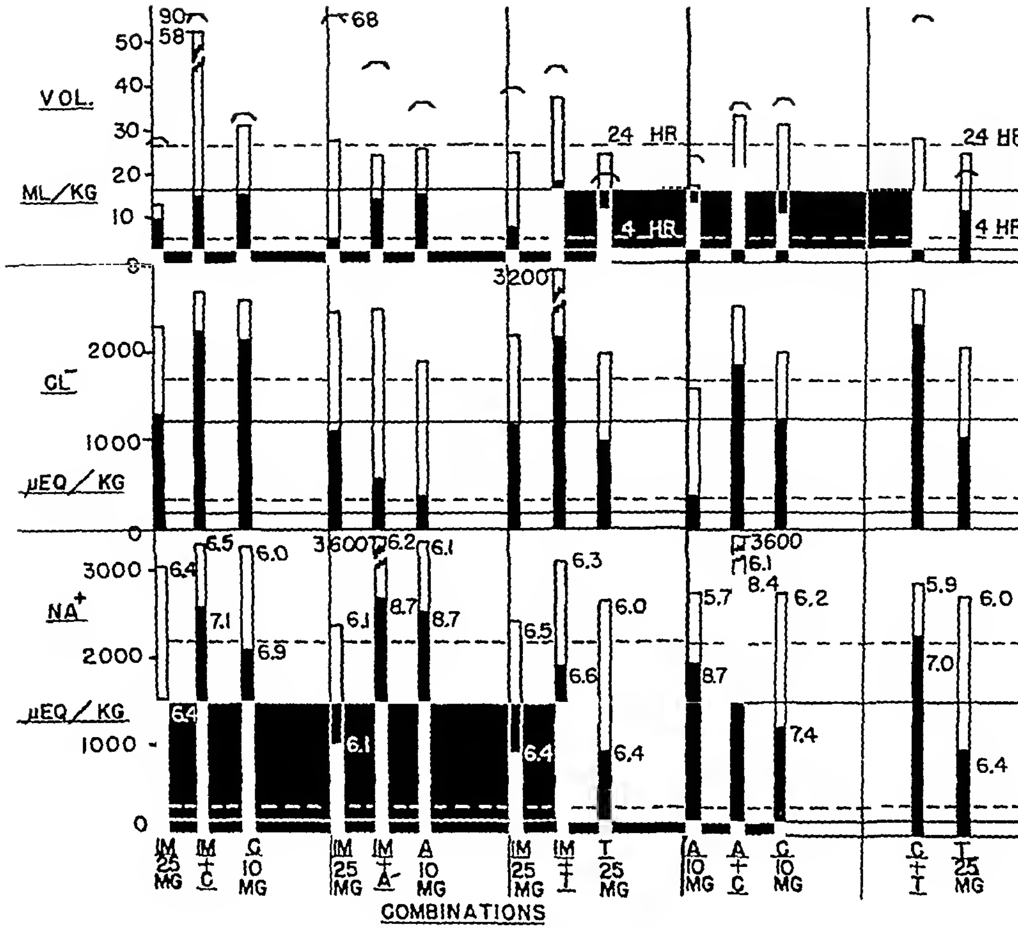

Fig. 8.—Results produced with combinations of diuretics given intraperitoneally with water *ad libitum.* IM (aminoisometradine), 25 mg. per Kg., + C (chlorothiazide), 10 mg. per Kg. IM (aminoisometradine), 25 mg. per Kg., + A (acetazolamide), 10 mg. per Kg. IM (aminoisometradine), 25 mg. per Kg., + T (8-chlorotheophylline), 25 mg. per Kg. A (acetazolamide), 10 mg. per Kg., + C (chlorothiazide), 10 mg. per Kg. C (chlorothiazide), 10 mg. per Kg., + T (8-chlorotheophylline), 25 mg. per Kg.

To enhance the activity of chlormerodrin it was theorized that brombenzene *(11)* would reduce the amount of —SH groups available to react with the mercury compound and thus enable the diuretic to become more effective (12). However, when rats were fed a diet containing 4% brombenzene or given 100 *mg.* per Kg. daily by stomach tube, the diuretic activity of the mercurial was not increased. A dose of *n*-ethyl-maleimide, 1 mg. per Kg. orally, likewise did not assist activity.

Since male animals had been found to give somewhat better indications of diuretic activity (5), it was believed that treatment of males or females with the opposite sex hormones might intensify either diuresis or nondiuresis. Therefore, recently weaned rats of both sexes were given diets providing 500 μg. of methyltestosterone or 50 μg. of estradiol per animal per day for six weeks before diuretic testing The results gave only slight suggestions that the female hormone decreased diuresis in both sexes when treated with acetazolamide, aminoisometradine, or chlormerodrin.

SUMMARY

Patterns of urinary excretion produced by diuretics administered to both excessively hydrated and untreated rats have been described. Excessive hydration was found to be unnecessary. The diuretic actions found could be divided into two categories; carbonic anhydrase inhibitors (acetazolamide), which rapidly increased the urine volume and sodium excretion with a high pH, and saluretic agents: (*a*) Pyrimidine (aminoisometradine), purine (aminophylline), and chlorothiazide types which also rapidly increased the volume with saluretic activity but not elevating the pH to the above extent. (*b*) Mercurials (chlormerodrin) which had little *immediate* activity but produced a delayed urine output with somewhat depressed chloride values. (*c*) Mis-

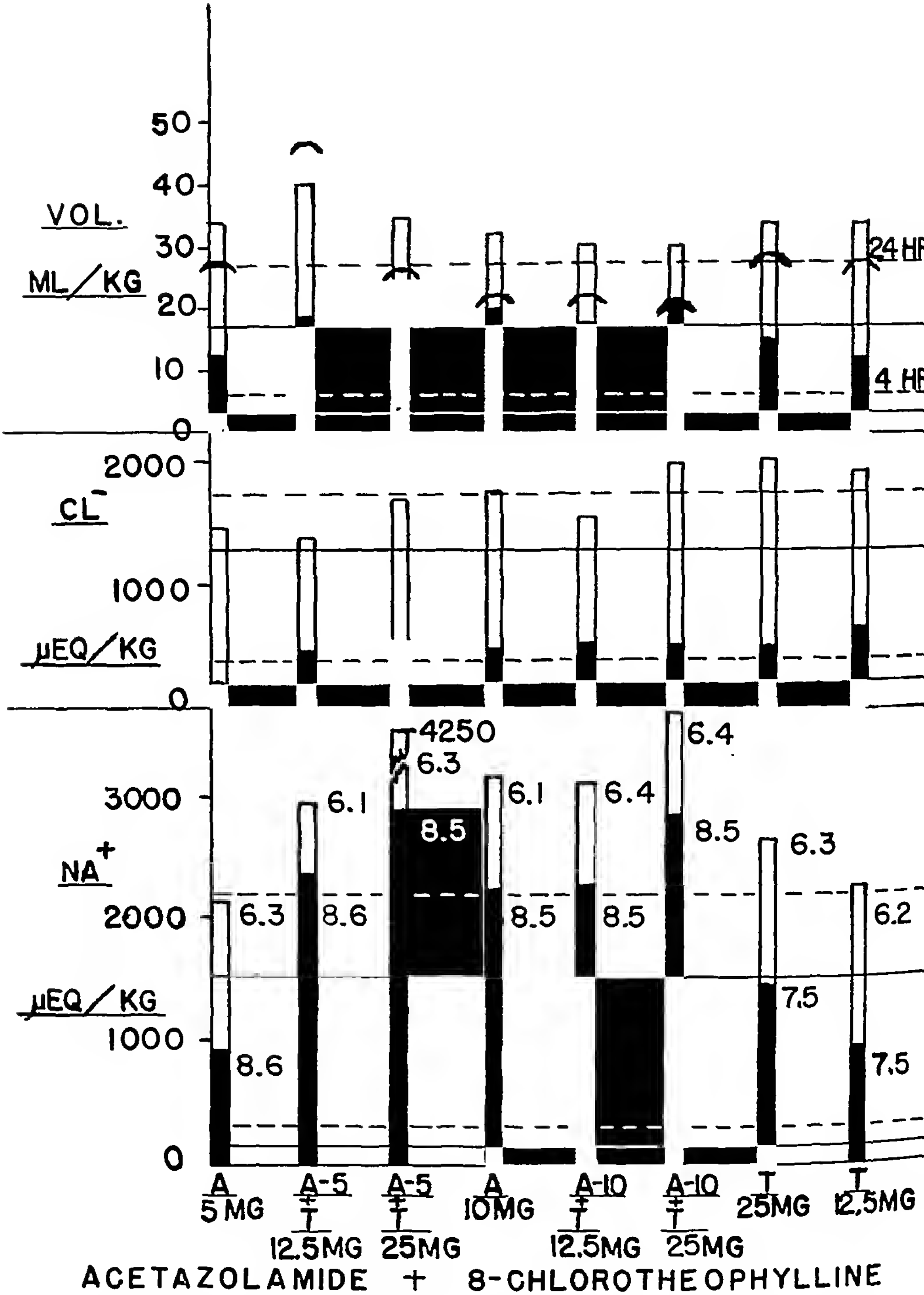

Fig. 9.—Results produced with varied combinations of acetazolamide (A) and 8-chlorotheophylline (T).

cellaneous types (triazines, etc.) which had immediate saluretic activity within narrow dose ranges. Combinations of these proved interesting with possibly some potentiation of activities observed, especially with acetazolamide and 8-chlorotheophylline. Due to the inactivity of —SH binding agents in enhancing the diuretic activity of chlormoren driit is suggested that this mode of action of mercurials be considered as still open to question.

TABLE II.—EXAMPLES OF COMPOUNDS TESTED[a]

		Urine Totals for 4 Hours				Urine Totals for 20 Hours			
	Dose, mg /Kg	Volume, ml /Kg	Na+, micro-equiv / Kg	Cl−, micro equiv / Kg	pH	Volume, ml /Kg	Na+, micro-equiv / Kg	Cl−, micro-equiv / Kg	pH
Control,		3 47	149	185	6 24	13 71	1,358	1,049	6 48
B W 49-335,	10	7 76	568	552	5 93	33 2	1,780	1,220	6 61
2,8-dichloro-6 aminopurine	100	18 88	1,408	1,648	6 52	107 2	5,580	4,040	6 62
B W 57-10, 4-(sec-butylamino)-pyrrolo [2,3-δ]-pyrimidine	10	8 00	272	236	6 65	51 8	2,640	1,640	6 71
	100	17 40	1,048	388	7 47	14 4	1,600	1,440	6 12
B W 55-325,	25	8 08	364		7 12	43 4	1,300		6 86
2-amino-4-hydroxy-5-methyl-6-phenyl-pyrimidine	50	10 76	1,020		6 18	65 6	1,860		6 66
	250[b]	1 24	68		5 66	34 6	3,940		6 51
B W 53-309,	10	9 72	856	720	6 29	77 8	2,040	1,460	6 39
N-phenyl-N′-acetylurea	100		no urine			57 8	9,240	5,320	6 31
B W 57-205,	2 5	7 72	440	572	6 17	23 0	2,020	1,560	6 62
2-amino-4-(2,4-dichloro-anilino)-1,3,5-triazine·HCl	5 0	10 40	736	752	6 17	29 6	1,880	1,160	6 45
	10 0	8 52	708	708	6 65	20 8	1,900	1,360	6 28

[a] These compounds were supplied by the Chemotherapeutic Section of the Wellcome Research Laboratories, Tuckahoe N Y [b] One rat died at this dose

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Salts of *p*-Acetamidobenzoic Acid*

By ANDREW LASSLO, CARL C. PFEIFFER, and PAULINE D. WALLER

_p_-Acetamidobenzoic acid yields atmospherically stable salts with amines which have a tendency to give hygroscopic products with conventionally used acid components. The physical properties of _p_-acetamidobenzoic acid salts, combined with the innocuous nature and absorption characteristics of the acid, render the latter valuable as a salt-forming agent for pharmaceutical preparations. Procedures for the preparation of _p_-acetamidobenzoic acid salts are reported.

RECENTLY, Pfeiffer (1) and his co-workers reported the effect of 2-dimethylaminoethanol (DMAE) upon the central nervous system and called attention to its potentialities in the treatment of mental disorders In connection with an extensive clinical study, we became interested in preparing a stable nonhygroscopic salt of DMAE which would facilitate adequate standardization of oral administration and permit satisfactory correlation of the data obtained by the participants in the investigation Of several possible salts, the salicylate, *d*-tartrate, *d*-pantoate, and *p*-acetamidobenzoate were chosen for evaluation. Salicylate and tartrate salts are frequently employed in pharmaceutical preparations, *d*-pantoic acid is a component of the vitamin pantothenic acid, and *p*-acetamidobenzoic acid is the normal metabolite of *p*-aminobenzoic acid.

In a study of the physical properties of these four salts, DMAE *p*-acetamidobenzoate[1] was found to be the least hygroscopic (Table I). In regard to toxicity, one could hardly find a more

* Received February 4 1959, from the Department of Pharmacology, Division of Basic Health Sciences, Emory University, Atlanta 22, Ga
This investigation was supported by a grant from the Geschickter Fund for Medical Research
Patent applied for

[1] Marketed as Deaner by the Riker Laboratories, Inc, Los Angeles, Calif.

TABLE I.—COMPARATIVE EFFECT OF AVERAGE ATMOSPHERIC CONDITIONS

Salt	Weight[a] of Sample, mg.	Period of Exposure in a Nonairconditioned Room, Hours	Change in Weight mg.	%
2-Dimethylaminoethanol *d*-pantoate	500	64	+132.2	+26 44
2-Dimethylaminoethanol salicylate[b]	1,000	64	+154 2	+15.42
2-Dimethylaminoethanol *d*-tartrate (bitartrate)[b]	1,000	64	+57.7	+5.77
2-Dimethylaminoethanol *p*-acetamidobenzoate	500	64	−1 4	−0.28

[a] Prior to evaluation, samples were dried at 25°/0.07 mm. Hg for twenty-eight hours
[b] Courtesy Riker Laboratories, Inc., Los Angeles, Calif.

innocuous acid component. Cantarow and Schepartz (2) report its presence in normal blood and urine, and consider it a metabolite of folic acid (for details on the metabolism of *p*-acetamidobenzoic acid in humans see Zehender's work (3)). These properties render *p*-acetamidobenzoic acid particularly valuable as a salt-forming agent for pharmaceutical preparations; especially so, when administration over an extended period of time is necessary. The efficacy of *p*-acetamidobenzoic acid acquires additional emphasis, if we consider the disadvantages one may encounter with salicylic and tartaric acids. The toxic properties of salicylic acid and the so-called "salicylates" are well known; diminished caloric intake may predispose a person to serious intoxication with no more than usual doses of "salicylates." Some asthmatic patients are sensitive to minute doses of salicylate. There is evidence (4) that salicylates may accumulate, if administered over a prolonged period of time; this becomes of particular concern, if renal function is below normal. In regard to tartaric acid salts, questions have been raised as to the nature and degree of their absorption from the human intestine (5, 6); actually several tartrates are known as cathartics, and cathartic action argues against much absorption from the intestinal tract (7). More specifically, Harington (8) states that orally administered hexamethonium bitartrate was always more poorly absorbed than other orally administered hexamethonium salts. In accordance with these findings, Pfeiffer (9) also observed a substantial decrease in the effectiveness of orally administered DMAE when given in the form of its *d*-bitartrate. It is interesting to note that Hagen (10) reported loss of *l*-epinephrine activity in stimulating oxygen consumption of mouse parotid glands incubated *in vitro* in a calcium-free medium, when the base was administered as the *d*-bitartrate salt.

The desirable properties of DMAE *p*-acetamidobenzoate suggested the use of *p*-acetamidobenzoic acid in the preparation of salts of other bases which have a tendency to yield hygroscopic salts with conventionally used acid components. The new salts are listed in Table II.

EXPERIMENTAL

The compounds listed in Table II were prepared by the following procedures:

Procedure A.—2-Dimethylaminoethanol *p*-Acetamidobenzoate.—Forty grams (0.223 mole) of *p*-acetamidobenzoic acid was dissolved in 500 ml. of boiling absolute methanol. Heating was discontinued, and a solution of 19.9 Gm. (0.223 mole) of dimethylaminoethanol in 100 ml. of absolute methanol was added with mechanical stirring, as fast as the exothermic nature of the reaction permitted. The resulting solution was allowed to cool to room temperature and was suction-filtered through Celite (Johns-Manville filter aid). The filtrate was poured into 500 ml. of anhydrous ethyl ether seeded[2] with a few crystals of DMAE *p*-acetamidobenzoate; crystallization ensued immediately. The product, a white crystalline material, was dried at 25°/0.08 mm. Hg for fifteen hours (48.4 Gm., 80.9% yield). It may be recrystallized from absolute ethanol–ethyl acetate, and dried as indicated above. The salt is readily soluble in water and is very stable over a wide range of atmospheric conditions. During its characterization, we have found that it will decompose when exposed to high temperatures under vacuum (100°/0.08 mm. Hg) and yield free *p*-acetamidobenzoic acid (72.5 mg. of the salt yielded quantitatively 47.8 mg. of the acid after four hours at 100°/0.08 mm. Hg).

Choline *p*-acetamidobenzoate remained unaffected under the same conditions; this was probably due to the increased basicity of choline.

Procedure B.—Nicotine *p*-Acetamidobenzoate.—Twenty grams (0.112 mole) of *p*-acetamidobenzoic acid was dissolved in 300 ml. of boiling absolute methanol. Heating was discontinued and 20.0 Gm. (0.124 mole) of nicotine was added. The resulting solution was thoroughly mixed, suction-filtered through Celite, and the solvent was removed under reduced pressure (max. pot temp. 50°). The viscous residue was mechanically agitated in 200 ml. anhydrous ethyl ether; it gradually turned into a pink powder. The crude product was dried at 25°/0.1 mm. Hg for one hour (32.2 Gm., 84.5% yield). Upon recrystallization from absolute ethanol–ethyl acetate, the pure salt was obtained in the form of pink crystals, after a first crop of unreacted *p*-acetamidobenzoic acid; it was dried as indicated above. The salt is readily soluble in water and is

[2] Three to six milliliters of filtrate added to 10 ml. of anhydrous ethyl ether yielded crystals within ten to fifteen minutes at room temperature.

TABLE II —PHYSICAL AND ANALYTICAL DATA

Compound	Method of Prepa ration	Melting Point, °C [a]	Optical Properties	Empirical Formula	Analyses[b] C, % Calcd	C, % Found	H, % Calcd	H, % Found	N, % Calcd	N, % Found
2 Dimethylamino ethanol *d* pantoate	C		n_D^{27} 1 4757 $[\alpha]^{28}_D{}^5$ +12 9° (c = 13 4, in abs methanol)	$C_{10}H_{23}NO_5$	50 61	50 68	9 77	9 51	5 90	6 06
2 Dimethylamino ethanol *p* acetamido benzoate	A	159 0–161 5		$C_{13}H_{20}N_2O_4$	58 19	58 20	7 51	7 64	10 44	10 5
2 Diethylaminoethanol *p* acetamidobenzoate	B	129 0–131 0		$C_{15}H_{24}N_2O_4$	60 78	60 79	8 16	8 21	9 46	9 46
Triethylamine *p* acetamidobenzoate	B	137 0–138 0		$C_{15}H_{24}N_2O_3$	64 25	64 10	8 63	8 55	10 00	9 93
Choline *p* acetamido benzoate	A	231 6–232 2		$C_{14}H_{22}N_2O_4$	59 55	59 56	7 86	7 76	9 93	9 68
Nicotine *p* acetamido benzoate	B	Decomp at 134–137	$[\alpha]^{23}_D$ +14 0° (c = 8, in H_2O)	$C_{19}H_{23}N_3O_3$	66 84	66 96	6 79	6 86	12 31	12 15

[a] Uncorrected [b] Analyses by Drs G Weiler and F B Strauss, Oxford, England

stable under atmospheric conditions; it will decompose when exposed to high temperatures under vacuum (100°/0 1 mm. Hg)

Procedure C.—2-Dimethylaminoethanol *d*-Pantoate.—To an aqueous solution (50 ml) of *l*-pantoyl lactone (5 07 Gm., 0 039 mole), 4 89 Gm (0 028 mole) of barium hydroxide was added and the resulting solution was heated at 74–81° for three hours Subsequently, excess barium was precipitated as the carbonate by bubbling a stream of carbon dioxide through the hot solution, and the precipitate was filtered off with Celite The filtrate, containing 0 019 mole of barium pantoate, was cooled to room temperature and added to an aqueous solution (26 ml) of 0 019 mole of dimethylaminoethanol sulfate (3 39 Gm. of dimethylaminoethanol neutralized with sulfuric acid to pH 6 8) The reaction mixture was thoroughly mixed and allowed to stand overnight The precipitated barium sulfate was filtered off with Celite, and the water was removed under reduced pressure (max pot temp 30°) The viscous residue was dissolved in about 300 ml of absolute methanol, the solution was filtered through Celite, and the solvent was removed under reduced pressure The residue was redissolved in another 300 ml of absolute methanol, the solution was refrigerated for about fifty hours, filtered through Celite, the solvent was removed under reduced pressure, and the residue was subjected to a vacuum of 0 07 mm Hg at 25° for a period of forty-three hours The product (7 5 Gm , 83 2% yield) was a clear, yellow, viscous liquid, which has retained its original consistency over a period of ten months; all attempts to crystallize the salt proved unsuccessful.

SUMMARY

The suitability of nonhygroscopic *p*-acetamidobenzoic acid salts for pharmaceutical preparations was pointed out The preparation of the *p*-acetamidobenzoic acid salts of nicotine, choline, 2-dimethylaminoethanol (DMAE), 2-diethylaminoethanol, and triethylamine, and that of the *d*-pantoic acid salt of DMAE was described.

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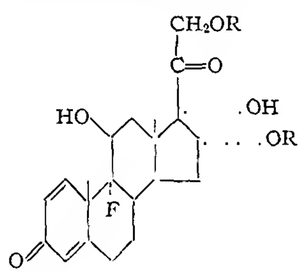
16α-Hydroxy Steroids I*

Characterization of Triamcinolone

By LELAND L. SMITH and MURRAY HALWER

Triamcinolone and triamcinolone 16α,21-diacetate have been characterized by ultraviolet absorption spectra, infrared absorption spectra, absorption spectra in sulfuric acid, polarography, reduction of tetrazolium blue, and by Porter-Silber chromogens. The use of absorption spectra, polarography, and colorimetry for quantitative determination of bulk triamcinolone and of triamcinolone in tablets is discussed. Some new chemistry of 16α-hydroxylated steroids is reviewed.

TRIAMCINOLONE (9α - fluoro - 11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) is a new potent corticosteroid of clinical importance (1–3). Triamcinolone is currently marketed in this country as the alcohol, although the 16α,21-diacetate has been tested clinically (4). Characterization of the hormone has been limited to brief accounts in the chemical journals (5–7) and in the patent literature (8). A more detailed characterization of both triamcinolone and triamcinolone diacetate is the subject of this report.



I. R=H   II R=COCH$_3$

Triamcinolone (I) and its diacetate (II) are distinguished structurally from other commercially important corticosteroids by the presence of the 16α-hydroxyl group, the $\Delta^{1,4}$-3-ketone, 11β-hydroxyl,9α-fluoro, and dihydroxyacetone side-chain features being common to other hormones. The profound biological effect of the 16α-hydroxyl group in the steroid molecule is accompanied by some altered physical and chemical properties, as certain typical reactions of corticosteroids are influenced by the introduction of the 16α-hydroxyl.

Absorption spectra in alcohol and in alcoholic alkali (9) (Tables III and IV) and in sulfuric acid (8, 10) (Table V), characteristic polarographic reduction behavior (11, 12), and infrared absorption spectra (Figs. 1 and 2) in the 6.0–6.25 μ region (11, 13) exhibited by triamcinolone and by its diacetate are typical of $\Delta^{1,4}$-3-ketosteroids of this series. Triamcinolone forms a diacetate (5) and a bis-2,4-dinitrophenylhydrazone (8). The usual color tests (spot tests on filter paper) used for the detection of Δ^4-3-ketosteroids, such as Bush's alkaline fluorescence (14), 2,4-dinitrophenylhydrazine (15), *p*-phenylenediamine phthalate (16), etc., fail with triamcinolone as they do with other $\Delta^{1,4}$-3-ketones. Isonicotinic acid hydrazide has proved of use in detection of triamcinolone (17), the reported failure of the reagent for detecting other $\Delta^{1,4}$-3-ketosteroids notwithstanding (18). Triamcinolone fails to react with Girard P reagent in the usual manner, such behavior again being expected of $\Delta^{1,4}$-3-ketones (19). This failure to react with Girard P reagent permits separation of triamcincolone from related Δ^4-3-ketones.

The presence of the 16α-hydroxyl group adjacent to the dihydroxyacetone side-chain affects the response of the steroid in several analytical procedures and enables one to prepare derivatives characteristic of planar *cis*-diols. Thus 16α,17α-acetonides (7, 20) and 16α,17α-cyclic borates (21) have been reported. Although triamcinolone has the anticipated reducing action toward tetrazolium blue on spot testing on filter paper, instrumentally the response to tetrazolium blue (22) is about 70% greater than that for non-16α-hydroxylated analogs. This property is suggested as characteristic of 16α,17α,21-trihydroxy-20-ketones (*cf.* Table I). The cyclic 16α,17α-diol derivatives respond more like the non-16α-hydroxylated parent steroids, the 16α,17α-acetonide of triamcinolone having but 40% of the response of triamcinolone, the 16α,17α-cyclic borate preparations having 60–65% of the response.

Triamcinolone does not respond to the Porter-Silber test (23) in the anticipated manner (*cf.* Table II). The response of triamcinolone and a variety of other 16α,17α,21-trihydroxy-20-ketones is depressed to about 10% of that of the non-16α-hydroxyl analogs. This poor response

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* Received November 21, 1958, from the Chemical Process Improvement Dept., Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The authors wish to acknowledge the assistance of Messrs. Michael Marx and Harold Mendelsohn in the preparation of triamcinolone and triamcinolone diacetate standards. Ultraviolet absorption spectra were obtained under the direction of Mr. Walter Muller, infrared spectra were kindly supplied by Mr. William Fulmor.

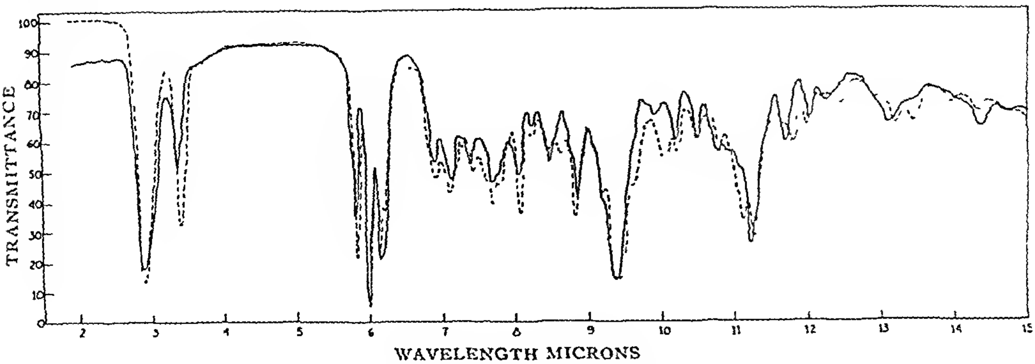

Fig 1 —Infrared absorption spectra of triamcinolone polymorphs in KBr disks. ——— Type I, - - - - Type II.

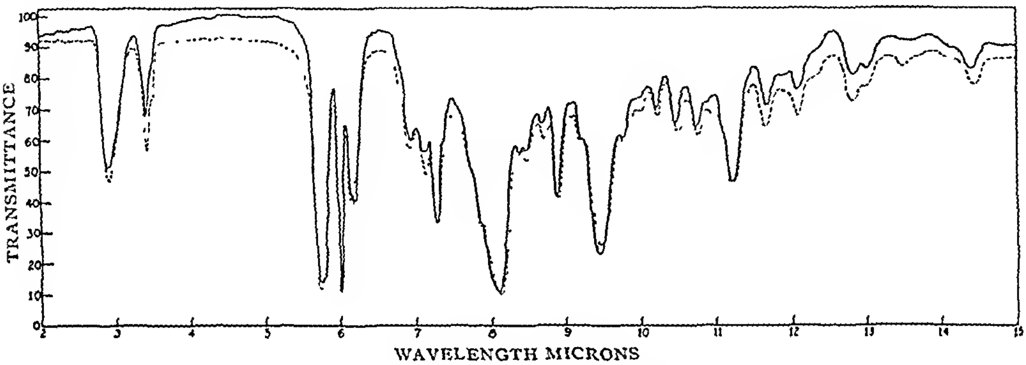

Fig. 2—Infrared absorption spectra of triamcinolone 16α,21-diacetate polymorphs in KBr disks. ——— Type I, - - - - Type II.

obviates the use of the Porter-Silber assay in biological systems where non-16α-hydroxylated steroids are also to be measured.[1]

Triamcinolone and the related 1,2-dihydro compound (9α-fluoro-11β,16α,17α,21 - tetrahydroxy-4-pregnene-3,20-dione) fail to give positive tests for the 17α-hydroxyl group with the sulfuric acid-phosphorus pentoxide reagent of Steyermark and Nowaczynski (24). No selective absorption occurs in the 400–600 mμ region. The generality of this test must remain questionable on other grounds, as neither prednisone, prednisolone, nor 9α-fluoroprednisolone give selective absorption in the 400–600 mμ region. Since 9α-fluorohydrocortisone does have selective absorption at *ca.* 550 mμ (E 1%, 1 cm. 159), both the 1,2-double bond and the 16α-hydroxyl may interfere with this color test.

Triamcinolone isomerizes under a variety of conditions to form a tetrahydroxy-diketone; such isomerization is also observed for at least one other 16α-hydroxylated steroid, 9α-fluoro-11β,16α,17α,21-tetrahydroxy-4-pregnene-3,20-dione. These isomers are more polar than the parent steroid, and may be expected to interfere with certain instrumental assays for triamcinolone. The isomerization of triamcinolone will be the subject of a later report.

TABLE I.—RELATIVE REDUCING POWER WITH TETRAZOLIUM BLUE OF 16α-HYDROXYLATED STEROIDS

Parent Steroid	Reducing Power of Parent Non-16α-Hydroxylated Steroids $E_{1\,cm}^{1\%}$ 510–530 mμ	Reducing Power of 16α-Hydroxylated Analog $E_{1\,cm}^{1\%}$ 510–530 mμ
11β,17α,21-Trihydroxy-4-pregnene-3,20-dione (hydrocortisone)	610	1,050
9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione (9α-fluorohydrocortisone)	610	1,040
11β,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione (prednisolone)	640	1,060
9α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione (9α-fluoroprednisolone)	620	1,050

The tetrazolium blue determinations were carried out as described in the *Experimental* part, read vs a reagent blank after thirty minutes standing at room temperature

[1] The failure of triamcinolone to undergo the Porter-Silber reaction has recently been reported Walker, A and Schlunke, H P, *Experientia*, 15, 71(1959) The increased reducing power of triamcinolone towards alkaline ferricyanide, as compared with non 16 α hydroxylated steroids has also been noted Stephenson, N R, *Can J Biochem. and Physiol*, 37, 391(1959)

TABLE II.—RESPONSE TO PORTER-SILBER CONDITIONS OF TRIAMCINOLONE AND RELATED 16α-HYDROXYLATED STEROIDS

Parent Steroid Nucleus	Non-16α-Hydroxylated Steroid $\lambda_{max.}$	Non-16α-Hydroxylated Steroid $E^{1\%}_{5\ cm.}$	16α-Hydroxylated Steroid λ_{max}	16α-Hydroxylated Steroid $E^{1\%}_{5\ cm.}$
9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione	417 mμ	1,350	420 mμ	241
9α-Fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione	416 mμ	1,800	425 mμ	165
11β,17α,21-Trihydroxy-4-pregnene-3,20-dione	415 mμ	1,650	415 mμ	191
11β,17α,21-Trihydroxy-1,4-pregnadiene-3,20-dione	415 mμ	1,990	420 mμ (Inflection)	238
17α,21-Dihydroxy-4,9(11)-pregnadiene-3,20-dione	415 mμ	1,785	415 mμ	114

TABLE III. PHYSICAL PROPERTIES OF TRIAMCINOLONE

Property	Bernstein, *et. al.*, *J. Am. Chem. Soc.*, 78, 5693 (1956).	Thoma, *et. al.*, *J. Am. Chem. Soc.*, 79, 4818 (1957).	This Report
Melting Point (capillary)	260–262.5°[a]	248–250°	262–264° dec.[b]
Specific Rotation	$[\alpha]^{25}_{D}$ + 75° (acetone)	$[\alpha]^{23}_{D}$ + 71° (acetone)	$[\alpha]^{22}_{D}$ + 67.1° ±1.9° (MeOH)
λ_{max}^{EtOH} (ϵ)	238 mμ. (15,800)	....	239 mμ. (15,224 ± 122)[c,d]
Infrared Absorption	$\lambda_{max.}^{KBr}$ 3,388, 1,705, 1,660, 1,620, 1,604 cm.$^{-1}$	$\lambda_{max.}^{Nujol}$ 2.95, 5.85, 0.02, 6.16, 6.24, 11.26 μ	$\lambda_{max.}^{KBr}$ 2.95, 3.41, 3.45, 5.85, 6 01, 6.15, 6.21, 11.23μ
$\lambda_{max.}^{0.066\ N}$ NaOH in EtOH (ϵ)	....	....	240 mμ (14,500), 264 mμ (7,800 inflection), 310 mμ (2,270)[e]

[a] Bernstein, *et al.*, also report a sample of triamcinolone melting at 269–271°.
[b] Some preparations of triamcinolone have been observed to melt as high as 273–275° decompn.
[c] An average of six determinations with standard deviation.
[d] An inflection at 308 mμ (ϵ89) may be seen at high steroid concentration.
[e] Spectra taken three to five minutes after solution at room temperature. At 60° for one hour, 239 mμ (14,900), 264 mμ (7,800, inflection), 310 mμ (3,480), not changed after two further hours at 60°.

Analytical techniques which involve differential migration of the steroid will be described in the second paper of this series. Some useful procedures for identification of triamcinolone and for analysis of bulk and tableted triamcinolone are presented in this paper.

TRIAMCINOLONE

Identification.—Triamcinolone is characterized by the physical measurements of Table III. Triamcinolone may exist in at least two polymorphs, depending on the solvent used for crystallization, such behavior rendering the melting point of little use in identification. Characteristic infrared spectra of triamcinolone in the solid state serve to distinguish the polymorphs one from the other. Type I polymorph (obtained from pyridine, aqueous pyridine, etc.) and type II polymorph (from methanol or aqeuous methanol) are interconvertible. Whereas the usual infrared bands characteristic of $\Delta^{1,4}$-3-ketones (11, 13) are present in both types, the type I spectra does not differentiate the Δ^1- and Δ^4-bands, the two bands coalescing into one broad band at 6.18 μ, whereas type II spectra give well defined bands at 6.15 μ and 6.22 μ (Fig. 1). The fingerprint region also contains select points of difference. Mixed spectra (containing aspects of both type I and type II spectra) have been observed in some samples.

Although infrared identification of triamcinolone is preferable, absorption spectra in concentrated sulfuric acid may be used; the band positions at 260–263 mμ and at 310 mμ (Table V) distinguish triamcinolone from a host of other related steroids (10).

Of particular value is the distinctive time course of alteration of the sulfuric acid spectra. The steady increase in absorbance of the 375–390 mμ band together with its shift from 390 mμ to 375 mμ over twenty hours is not duplicated even by triamcinolone 16α,21-diacetate.

Determination.—Instrumental determination of triamcinolone may be made with a variety of techniques depending on the specificity required. Direct ultraviolet absorption measurements, direct polarographic measurements, and tetrazolium blue reducing power have served most uses. A preferred method combines polarographic determination (ring-A) together with determination with tetrazolium blue (side-chain). Where more specificity is required, chromatographic separations must precede instrumental determinations.

Triamcinolone Purification.—A selected, repeatedly recrystallized batch of triamcinolone, m. p. 249–250°; $[\alpha]^{22}_{D}$ + 67.4° (MeOH); $\lambda_{max.}^{EtOH}$ 239 mμ (ϵ 14,900); was partitioned on a column prepared as follows: 600 Gm. of acid-washed Celite diatomaceous earth was moistened with 300 ml. of the lower phase of the mixture: dioxane (10 liters) : cyclohexane (6 liters): water (2 liters), then packed in 2–3 inch layers into a three-inch diameter column. The sample of 600 mg. of triamcinolone dissolved in 20 ml. of lower phase was slurried into 40 Gm. of Celite diatomaceous earth and the charge packed onto the column. Development of the column with the upper phase of the solvent mixture eluted triamcinolone at 6.2–6.9 hold-back volumes (HBV) (1 HBV was 900 ml.). The fraction was evaporated to dryness *in vacuo* and crystallized from aqueous pyridine and from pyridine/methanol/acetone/water, m. p. 262–264° decompn., ϵ 15,060. Final recrystallization from pyridine/acetone (2:3) diluted with water gave the standard sample, m. p. 262–264° decompn.; ϵ 15,224 ± 122; $[\alpha]^{22}_{D}$ + 67.1° (MeOH). The polarograph half-wave potential was −1.02 v. (*versus* saturated calomel electrode), the diffusion current, $i_D/C \pm \sigma$, at − 1.10 v. being 4.39 ± 0.04 μa./mg./ml., at −1.25 v. being 4.54 ± 0.02 μa./mg./ml.

TRIAMCINOLONE 16α,21-DIACETATE

Identification.—Triamcinolone 16α,21-diacetate is characterized by melting point behavior, optical rotation, etc., in Table IV. Here again melting point behavior is of limited use in identification, although the wide melting range is characteristic of triamcinolone diacetate. At least two polymorphs have been prepared, as evidenced by infrared spectra in the solid state. The crystalline forms are interconvertible by using the appropriate solvent. Type I polymorph (from methylene chloride, chloroform, etc.) and type II polymorph (from acetone) are typified by the spectra of Fig. 2. Infrared absorption spectra of the diacetate in chloroform have recently been published (25).

TABLE IV.—PHYSICAL PROPERTIES OF TRIAMCINOLONE 16α,21-DIACETATE

Property	Bernstein, *et al.*, *J. Am. Chem. Soc.* 78, 5693, (1956)	Thoma, *et al.*, *J. Am. Chem. Soc.*, 79, 4818 (1957)	This Report
Melting point (capillary)	158–235°[a]	179–180° with gas evolution	145–236°[b]
Specific Rotation	$[\alpha]_D^{25}$ + 22° (chloroform)	$[\alpha]_D^{23}$ + 28° (chloroform)	$[\alpha]_D^{22}$ + 22° (chloroform) + 63° (MeOH)
λ_{max}^{EtOH} (ε)	239 mμ (15,200)		239 mμ (15,270)
Infrared absorption (KBr disk)[c]	3,635, 3,440, 1,720, 1,674, and 1,630 cm^{-1}[d]		2.90, 3.40, 5.74, 6.00, 6.15, 6.19, (shoulder) μ
$\lambda_{max}^{0.06}$ N NaOH in EtOH (ε)			240 mμ (13,800), 265 mμ (7,150 inflection), 310 mu (2,000)[e]

[a] Bernstein, *et al.*, note occasional melting points of 186–188° with gas evolution and mention apparent solvation of the diacetate. Also m. p. 150–240° with loss of solvent is reported in U. S. pat. 2,789,118.
[b] The melting range for type II crystal, for type I, 185–232° decompn.
[c] Spectra in chloroform have been published, see (25).
[d] Proving the analytical sample of Bernstein, *et al.*, with the criteria of polymorphism described herein indicates a type I crystal.
[e] Spectra taken three to five minutes after solution at room temperature. At 60° for one hour, 240 mμ (14,500), 261 mμ (8,750, inflection), 310 mμ (3,280), not altered after two more hours at 60°.

Type I polymorph has a slightly different melting point behavior from type II, type I starting to melt later than type II (185–232° *versus* 145–235°). Type I polymorph has been obtained from chloroform, methylene chloride, acetone, acetone/petroleum ether, and benzene/petroleum ether. Type II has been obtained from acetone and from acetone/petroleum ether.

Identification of the diacetate may also be made using spectra in concentrated sulfuric acid (Table V). Characteristic spectra have also been obtained in 100% phosphoric acid (26). Bands at 260 mμ ($E_{1\,cm}^{1\%}$ 299), 290 mμ (213, inflection), 310 mμ (205 inflection) and 375 mμ (144) characterize the diacetate in 100% phosphoric acid.

Determination.—Instrumental determination of triamcinolone diacetate may be made using the same methods that were described for the alcohol.

TABLE V.—SELECTIVE ABSORPTION SPECTRA OF TRIAMCINOLONE AND TRIAMCINOLONE DIACETATE IN CONCENTRATED SULFURIC ACID

	Time After Solution	λ_{max} ($E_{1\,cm}^{1\%}$)	λ_{min} ($E_{1\,cm}^{1\%}$)
Triamcinolone	15 min	260 mμ (334)	220 mμ (63)
		310 mμ (172)	287 mμ (128)
		390 mμ (42)	345 mμ (16)
	2 hr	260 mμ (346)	218 mμ (71)
		310 mμ (176)	288 mμ (142)
		380 mμ (86)	342 mμ (34)
	20 hr	263 mμ (386)	220 mμ (80)
		310 mμ (176)	300 mμ (166)
		375 mμ (94)	340 mμ (40)
Triamcinolone 16α,21 diacetate	15 min	261 mμ (297)	220 mμ (59)
		308 mμ (136)	283 mμ (88)
	2 hr	261 mμ (297)	220 mμ (59)
		308 mμ (136)	283 mμ (88)
		380 mμ (35)	345 mμ (17)
	20 hr	260 mμ (302)	217 mμ (82)
		308 mμ (137)	285 mμ (109)
		375 mμ (187)	335 mμ (50)
		475–480 mμ (10) Infl	

Triamcinolone 16α,21-Diacetate Purification.—A selected sample of chromatographically purified triamincolone diacetate, m. p. 148–190° decompn., λ_{max}^{EtOH} 239 mμ (ε 15,400), homogeneous on papergrams, infrared spectra polymorph type I was recrystallized from acetone/petroleum ether (Skellysolve B), m. p. 145–200° decompn., ε 15,200, type II polymorph. Recrystallization from chloroform/petroleum ether yielded type I polymorph, m. p. 185–232° decompn., ε 15,000. Still further recrystallizations from acetone/petroleum ether and from acetone failed to alter the characteristic melting range. After drying *in vacuo* over refluxing toluene the sample melted 145–235° decompn., with ε 15,270, type II polymorph. The polarographic half-wave potential was −1.01 v., the diffusion current at −1.30 v. was 3.55 μa./mg./ml.

EXPERIMENTAL

Ultraviolet absorption spectra were obtained in absolute ethanol using the Cary Recording Spectrophotometer, Model 11S; molecular extinction coefficients were calculated from replicate measurements using the Beckman Spectrophotometer, Model DU. Optical rotations were determined on *ca.* 0.5% solutions in the appropriate solvent. Sulfuric acid spectra were determined according to published procedures (10, 27). Infrared spectra were obtained on solid samples as potassium bromide disks, using the Perkin-Elmer Model 21 double beam instrument.

Polarographic Solvent.—A solution of 0.1 *M* tetra-*n*-butylammonium hydroxide in 50% aqueous methanol is adjusted to pH 3.0 with phosphoric acid. The solution is stable indefinitely.

Polarographic Determination.—A 10-mg. sample of triamcinolone is weighed into a 10-ml. volumetric flask and 8 ml. of polarographic solvent is added. The mixture is shaken and warmed on a steam bath until solution is complete, cooled, and made to volume with polarographic solvent and mixed thoroughly. The solution is placed in the semimicro

polarographic cell and the capillary adjusted, etc The current-voltage relationship is determined up to about -1.4 v using the Leeds and Northrup Electrochemograph instrument at a suitable sensitivity setting The diffusion current is determined from the reading at -1.10 v (half-wave potential is -1.02 v) The purity of the sample is obtained by comparison of the observed diffusion current with that of a standard run at the same time The related Δ^4-3-ketosteroids exhibit a half-wave potential of *ca* -1.20 v

Tetrazolium Blue Reagent.—Five hundred milligrams of tetrazolium blue is dissolved in 100 ml of absolute ethanol The solution is filtered into an amber glass-stoppered bottle Ten milliliters of a 10% solution of tetraethylammonium hydroxide is diluted with 100 ml of absolute ethanol and filtered The two solutions are stored in the refrigerator during the course of a series of determinations In no case should the solutions be retained after two weeks, and fresh tetrazolium blue reagent made daily is recommended

Colorimetric Determination.—Ten milligrams of triamcinolone is weighed into a 10-ml volumetric flask, 8 ml of absolute ethanol is added, and solution effected by vigorous shaking. Heat may not be applied to aid solution at this point as decreased absorption in color intensity invariably results, probably due to isomerization of triamcinolone. When solution is complete the sample is made to volume with ethanol and mixed A suitable dilution is made to give about 40 μg /ml. of steroid A 1-ml aliquot of the 40 μg /ml solution is placed in a 10-ml volumetric flask, 1 ml of tetrazolium blue reagent is added, followed by 1 ml. of the 1% tetraethylammonium hydroxide solution, and the solution is diluted to the mark with ethanol After thirty minutes the absorbance is determined at 510–530 mμ (maximum *ca* 525 mμ) using the Beckman DU instrument *versus* a reagent blank similarly treated. Results are calculated with reference to standard triamcinolone run at the same time Using these techniques the standard deviation for a single determination was 0 0093, a series of 28 replicates gave E(5μg /ml)/1 cm (510 mμ) of 0 549 $\pm$ 0 009 for triamcinolone diacetate

Determination of Triamcinolone in Tablets.—A sample of ten 2-mg tablets of triamcinolone is pulverized in a mortar and triturated with a few milliliters of absolute ethanol The sample is transferred to a 100-ml. volumetric flask, made up to about 80 ml with absolute ethanol, and shaken on a rotary shaker for one hour After making to volume with ethanol and mixing, the solution is centrifuged until a clear supernatant is had

This solution is sampled for the several assays required. For polarographic analysis an aliquot containing 2–3 mg. of triamcinolone is transferred to a glass-stoppered flask and evaporated to dryness with gentle warming under a stream of air The residue is dissolved in the calculated volume of polarographic solvent and the analysis made by polarographic means as already described For colorimetric determination with tetrazolium blue, suitable dilutions are made of the clear ethanol solution and the colorimetric assay made directly without intermediate removal of the solvent. Ultraviolet absorption measurements may also be made on the clear ethanol solution, a suitable dilution to about 10 μg /ml. being made, and the absorbance at 239 mμ taken on the Beckman DU instrument.

Comparison of the three assays on 4-mg. tablets of triamcinolone gave polarographic 4.19 mg , spectrophotometric 4.16 mg., colorimetric (tetrazolium blue) 3 98 mg.

Porter-Silber Determination.—The directions of Porter and Silber (23) are followed, using ethanol solutions of the steroid at concentrations ranging from 1.7 to 12 μg /ml Three milliliters of steroid solution is mixed with 24 ml. of the phenylhydrazine reagent (65 mg of phenylhydrazine hydrochloride in 62 ml of concentrated sulfuric acid and 38 ml of water), heated at 60° for twenty minutes, cooled, and absorption spectra recorded over the range 380–500 mμ using a 5-cm. cell (*cf.* Table II).

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A Study of the Effect of Some Emulsifying Agents on Drug Release from Suppository Bases*

By CLYDE W. WHITWORTH and JOSEPH P. LAROCCA

Suppositories consisting of varying amounts of hydrogenated cottonseed oil, Tweens, Spans, and Arlacels were incorporated with a dye and tested for the percentage of dye release at time intervals. Most of the suppositories were tested for storage qualities and seven were subjected to *in vivo* tests. Several of the proposed bases appear to be very promising and compare favorably *in vivo* with a commercial brand of suppository now being widely used.

Suppositories, one of the oldest forms of medication, have been the object of much experimentation. This is due to the fact that to be practical and useful suppository bases must give satisfactory drug releases, have good appearance and storage qualities, be neutral and nonirritating, and be compatible with all medication.

Until recent years theobroma oil and a few other fatty substances were accepted as the most satisfactory suppository bases. These fat-type bases had many disadvantages. Eiler (1) points out that oil soluble drugs when incorporated in fat-type bases (unless emulsified) tend to be released very slowly from the fat. Ohmart (2) states also that a fat-type base hinders the action of water-soluble medicaments by coating the mucosa of the body cavity. On the other hand, water-soluble drugs tend to be more readily released for either local or systemic effect when incorporated into a water-miscible base.

Theobroma oil and other like substances were used as suppository bases mainly because they melted at body temperature. This particular property is assuming less importance in face of the latest research in this field. It is quite apparent that the nature of the base is a determining factor in the rate of release of a drug for systemic medication. Gross and Becker (3) reported several new suppostitory bases in which emulsifying agents increased the rate of drug release. Del Pozo (4) found that Lanette Wax and Aerosol OT increased the rate of dialysis of potassium iodide from cocoa butter *in vitro*. In studying the influences of suppository bases upon the rectal absorption of acetylsalicylic acid, Cacchillo and Hassler (5) reported good absorption from Carbowax bases, fair absorption from theobroma oil bases, and poor absorption from glycerinated gelatin bases. An excellent review of suppositories, both old and new, is given by Gross and Becker (3).

In this study the line of endeavor was to produce acceptable suppository bases consisting of hydrogenated cottonseed oil and some of the newer synthetic emulsifying agents. Hartman and LaRocca (6) conducted a study on the use of mixtures of completely and partially hydrogenated cottonseed oil as suppository bases, obtaining good results. Coto Flakes,[1] which is completely hydrogenated cottonseed oil has a melting range of 58 to 62° and an iodine number of approximately 6. Cotmar,[2] which is partially hydrogenated cottonseed oil, has a melting point of about 34° and an iodine number of about 70. The emulsifying agents used were Spans, Tweens, and Arlacels.[3] Spans and Arlacels are essentially partial esters of the common fatty acids, lauric, palmitic, stearic, and oleic, and hexitol anhydrides derived from sorbitol. They are lipophilic and are generally insoluble in water, but soluble in organic solvents. Tweens are polyoxyethylene derivatives of the Spans (7). They are hydrophilic. Schwartz, *et al.* (8), conducted investigations using patch tests on human subjects and found these emulsifying agents to be nonirritating. Feeding tests have also proved them harmless (7).

EXPERIMENTAL

About seventy-five different suppository bases were prepared during the course of this study. From the results of *in vitro* study seven bases were chosen to be studied *in vivo*, in an attempt to correlate the two methods of determining drug release. All were made by fusion of the constituents with a minimum amount of heat. The melting points were determined using the capillary rise method for class two materials as described in the *United States Pharmacopeia* XV (9). Table I shows the formulas for the most satisfactory suppositories and their melting points.

All the suppositories were observed at room temperature for at least one week and the appearance noted. Several of those thought suitable were subjected to further tests to determine storage qualities, in which one suppository was wrapped in aluminum foil and placed, along with an unwrapped

* Received March 13, 1958, from the Research Laboratories of the University of Georgia, School of Pharmacy, Athens.

[1] Supplied by the Proctor and Gamble Co.
[2] Supplied by the Proctor and Gamble Co.
[3] Supplied by the Atlas Powder Co.

TABLE I —PER CENT COMPOSITION AND MELTING RANGE OF FORMULAS

No Formula	Tweens 20	Tweens 40	Tweens 61	Tweens 81	Tweens 85	Spans 40	Spans 60	Spans 80	Arlacels 20	Arlacels 60	Arlacels 80	Arlacels 85	Cotmar	Coto Flakes	Melting Range, ° C
1	20		20										60	.	34-36
2		20	20										60		45-47
3		..	20			20							60		36-38
4			10										80	10	45-47
5				10									80	10	46-48
6					10								80	10	46-48
7			20										60	20	50-52
8				20									60	20	50-52
9					20								60	20	51-53
10							20						60	20	50-52
11		15	20										60	5	42-44
12			20					15					60	5	44-46
13			20			20							57 5	2 5	39-40
14						30							50	20	47-49
15			20						15				60	5	41-43
16			20							15			60	5	40-42
17			20								15		60	5	40-42
18			20									15	60	5	40-42
19													90	10	45-47

one, in an oven at 30° for approximately two months Not all the bases were subjected to the two months of oven testing, but all were tested for stability at room temperature In general, the proposed bases which were tested for storage compared favorably with several commercial suppositories

In Vitro Test for Drug Release and Storage Tests.—An exact amount of a dye, FD&C Red No 2, representing a water-soluble medicinal agent was incorporated into one suppository After twenty-four hours of refrigeration the suppository was ready for testing. The suppository containing the dye was placed in one percolator of water at 37° and a blank suppository in another Low speed stirrers maintained circulation At definite time intervals samples of water were withdrawn from each percolator and the dye release determined colorimetrically The reading of the blank was corrected each time for the effect of the base alone on the transmittance of light The actual percentage of dye released was determined from a graph previously prepared with known concentrations of the same dye The procedure is almost identical to that used by Hartman and LaRocca (6) who give the method in detail Table II shows the percentage of dye released for each formula tested.

In Vivo Testing of Suppositories.—A commercial brand of pentobarbital sodium suppository with a theobroma type base was tested and the time for onset of action recorded Thereafter, four of the proposed bases which showed good dye release were incorporated with pentobarbital sodium and tested in a like manner Two bases showing poor dye release were tested in the animals also Female rabbits were used in the study and 10 animals were used in each determination Loss of righting reflex was taken as the onset of action. Table III shows the results of the *in vivo* tests

DISCUSSION

Many different formulas for suppository bases were studied All contained hydrogenated cotton-

TABLE II —DYE RELEASE

Formula No	Per Cent Dye Release — Time in Minutes 2	4	6	8	10	12	14	30
1	42 5	42 5	43	43 5	44 3	44 3	44 3	44 3
2	25 7	38 5	38 5	38 5	38 5	39 3	39 3	39 3
3	2 9	2 9	20	32 8	37 6	39 4	39 4	41 8
4	2 8	4 7	11 8	17 9	23 9	26 4	30 7	42 1
5	1 0	4 3	7 1	11 1	12 5	14 3	15 5	21 4
6	2 1	3 9	7 9	12 5	16 1	21 4	23 5	38 5
7	1 0	2 1	2 1	2 1	3 6	4 3	4 3	5 7
8	0	2 0	3 4	5 7	7 0	7 5	8 0	10 0
9	2 8	2 8	4 3	5 0	5 0	5 7	6 0	12 5
10	0	0	0	0	0	0	1 4	2 0
11	5 1	5 1	7 5	14 6	17 5	28 0	33 2	55 4
12	1 4	3 9	4 3	16 0	25 0	41 0	42 8	71 4
13	0 7	0 7	31 4	45 7	45 7	45 7	45 7	45 7
14	0	0	0	0	0	0	0	1 0
15	2 8	5 7	24 6	35 7	40 7	46 5	53 5	57 8
16	0	0 7	2 1	2 1	5 0	10 0	28 5	47 8
17	1 4	3 2	7 1	11 4	18 5	22 0	25 0	38 5
18	2 1	3 5	5 7	14 2	17 1	20 7	22 2	52 2
19	0	0	0	0	0	0	0	0

TABLE III.—A COMPARISON OF THE RECTAL ABSORPTION FROM SOME PROPOSED BASES WITH A COMMERCIAL BRAND SUPPOSITORY IN RABBITS

Formula No	No of Rabbits Used	Average Time for Loss of Righting Reflex in Animals, min	Standard Deviation, min
3	10	11 5	±1 84
4	10	14 9	±3 82
10	10	Ineffective in 30 min	
11	10	8 3	±1 57
12	10	11 0	±1 89
14	10	Ineffective in 30 min.	
15	10	9 9	±2 27
X[a]	12	16 4	±5 28

[a] A commercial brand of suppository with a theobroma oil base

seed oil as the main constituent and varying amounts of surface active agents to increase the release of a drug as represented by a dye.

The percentages of completely and partially hydrogenated cottonseed oil were varied to control the melting range of the bases. The concentrations of emulsifying agents were varied to try to determine the amounts of these that would produce maximum dye release. The most desirable base would be one that contained a high percentage of hydrogenated cottonseed oil, which is relatively inexpensive, a high melting range for storage qualities, and a minimum amount of emulsifying agents that would produce acceptable drug release

As was expected, the bases containing the Tween products gave the best dye release and also proved effective *in vivo* This was probably due to the fact that the Tweens are hydrophilic and the dye and drug used were water soluble The Span products gave somewhat less effective dye release Some formulas containing both Spans and Tweens gave the best dye release Four of the bases showed better than 50% dye release in thirty minutes The *in vivo* tests compare seven proposed formulas with a commercial suppository as to onset of action

When the *in vivo* and *in vitro* results are compared, the average times for loss of righting reflexes in the rabbits corresponds to a dye release of 31 to 40% in four of the five bases which were most effective *in vivo* Two bases which gave poor dye release were ineffective *in vivo*

The most effective base in the *in vivo* test gave a loss of righting reflex in approximately half the time of the commercial suppository. The action of the commercial suppository was somewhat erratic as shown by the standard deviation

The results in this study cannot be compared precisely with other studies using the same method of *in vitro* tests because the composition of formulas are not the same. However, certain comparisons can be made. Gross (3) found that certain surface active agents when incorporated into theobroma oil increased dye release Hartman and LaRocca (6) found almost no dye release from bases containing only hydrogenated cottonseed oil; these results were duplicated in this study. In general, other studies indicate that emulsifying and surface active agents increase drug release.

The results indicate that dye release is highest in bases that contain 35–40% of the emulsifying agents and have a melting range of less than 48° Above this melting range the dye release and effectiveness *in vivo* drops off sharply

All the suppositories reported here had a smooth, pleasing appearance and were quickly and easily made

SUMMARY AND CONCLUSION

Nineteen suppository bases are given Several of the bases show good release of a water-soluble active ingredient both *in vivo* and *in vitro*

Judging from the dye release tests it seems that those bases containing 35–40 per cent of certain of the emulsifying agents gave best release. The Tween products seem ideally suited as ingredients in suppository bases and several of the bases appear to be superior to theobroma oil as a systemic suppository base

Seemingly, more effort should be made to correlate *in vivo* and *in vitro* testing of suppositories

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Antimicrobial Substances from Seeds*

By JASPER C. MARUZZELLA and MARTIN FREUNDLICH

One hundred and ninety-five extracts were prepared from 39 seeds of drug origin and screened *in vitro* against the following test organisms: *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Mycobacterium smegmatis, Candida albicans, Erwinia caratovora,* and *Streptomyces Venezuelae.* The di-ethyl ether and acetone soluble fractions were found to possess greater antimicrobial activity than the ethyl alcohol, *n*-butyl alcohol, and water-soluble fractions. Sixteen of the 39 seeds were found to have no antimicrobial activity. Extracts of cardamom (green), tonka angostura, and celery were found to have antimicrobial activity on at least 6 of the 7 test organisms as well as against 12 additional microorganisms. Concentration of the extracts to less than one-half the original volume did not increase antimicrobial activity.

The screening of higher plants for antimicrobial substances has been an active area of investigation for the past two decades. Among the more complete of these studies are those of Osborn (1), Lucas and Lewis (2), Huddelson, *et al.* (3), Sanders, *et al.* (4), Carlson, *et al.* (5), and Okazaki, *et al.* (6–11). These investigators have tested more than 4,000 higher plants with about 20 per cent exhibiting some antibacterial activity. Other preliminary surveys are those of Atkinson and Rainsford (12), Hayes (13), Carlson, *et al.* (14), and more recently Masilungan, *et al.* (15), Atkinson (16), Ferenczy and Gracza (17), and Hasegawa, *et al.* (18). The parts of the plants examined for activity were: leaves, stems, roots, barks, flowers, entire plant, fruits, and occasionally seeds. Why seeds should be somewhat neglected in these surveys is not known. This does not mean that seeds have not been investigated, for indeed they have (19–25).

Similarly, screening for antimicrobial substances from plant drugs has been investigated (26–31) with little emphysis placed on seeds. This investigation therefore is concerned with extracting antimicrobial substances from seeds of drug origin.

MATERIALS AND METHODS

Test Organisms.—The bacteria employed were: *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Mycobacterium smegmatis, Erwinia caratovora, Streptomyces Venezuelae, Sarcina lutea, Bacillus subtilis, Neisseria perflava, Salmonella cholerae suis, Salmonella typhi murium,* and *Proteus vulgaris.* The fungi used were: *Candida albicans, Trichophyton mentagrophytes, Microsporum canis, Helminthosporium sativum, Cryptococcus rhodobenhani, Penicillium digitatum,* and *Epidermophyton interdigitale.* All of the bacteria were cultivated in a nutrient broth medium consisting of: polypeptone 5 Gm., beef extract 3 Gm., and distilled water to make 1 L. For the nutrient solid medium 15 Gm. of agar was added to this formula. The fungi were cultivated on Sabouraud's dextrose broth and agar (Difco).

Preparation of Extracts.—The solvents used for extracting antimicrobial substances from the seeds were: distilled water, ether, acetone, alcohol, and *n*-butyl alcohol. The extraction procedure was conducted as follows: Twenty grams of dry seeds was added to 50 cc. of solvent and allowed to soak for one hour. The material was placed into a Waring Blendor and macerated for two minutes, then filtered through filter paper.

Method of Testing.—The filter paper disk method of Vincent and Vincent (32) was used to determine the presence of antimicrobial activity. In this method 1 cc. of a twenty-four hour broth culture of bacteria was placed into Petri dishes with approximately 10 cc. of nutrient agar. In the case of the fungi, 1.5 cc. of a two to three-day old culture were placed into Petri dishes together with about 10 cc. of Sabouraud's dextrose agar. Filter paper disks (6.35 mm. diameter) were saturated with the extract and allowed to air dry. This procedure was repeated so that each disk contained four applications of the extract. The disks thus prepared were placed on the surface of the seeded agar. All dishes with bacteria were incubated at 37° for twenty-four hours except *E. caratovora* (forty-eight hours at room temperature) and *S. Venezuelae* (twenty-four hours at room temperature). All of the dishes with fungi were incubated at 37° for three days except *H. sativum* which was incubated for two days at room temperature. Zones of inhibition surrounding the disks were measured with a mm. ruler and an illuminated Quebec colony counter. Six disks were prepared for each extract and tested against each organism. Zones of inhibition recorded represent a mean value of six readings.

Additional extracts were prepared in the same manner as described above but the final volume of these extracts was concentrated (by evaporation) to less than one-half its original volume. These concentrated extracts were also tested for antimicrobial activity.

RESULTS AND DISCUSSION

One hundred and ninety-five extracts were prepared from 39 seeds and screened against seven test

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This paper is based in part on a thesis submitted by Martin Freundlich to the Graduate School of Long Island University in partial fulfillment of the requirements for the degree of Master of Science.

All of the seeds used in this investigation were generously supplied by S. B. Penick Company, New York City.

TABLE I.—ANTIMICROBIAL ACTIVITY OF SEED EXTRACTS

Seeds	*E. coli*	*S. aureus*	*S. marcescens*	*M. smegmatis*	*C. albicans*	*E. caratovora*	*S. Venezuelae*
	Zone of Inhibition in mm[a]						
Ambrette[b] (wa)	0[c]	0	0	2	0	0	0
Angelica (ac)	0	2	0	2	3	2	5.5
Angelica (ba)	0	2	0	2	3	2	5.5
Anise (ac)	0.5	2	1.5	3	4	1	4
Annatto (ee)	0	1	0	0	0	0	0
Annatto (ac)	0	1	0	0	0	0	0
Annatto (ea)	0	1	0	0	0	0	0
Annatto (ba)	0	1	0	0	0	0	0
Asparagus (wa)	2	2	0	1.5	0	1	2
Caraway (ea)	0.5	0.5	0.5	0	2	0.5	4
Caraway (ba)	0.5	0.5	3	0	2	0.5	2
Cardamom, green (ee)	4	2	0.5	5	4	0.5	6
Cardamom, green (ac)	4	3.5	0.5	5.5	3	0.5	4.5
Cedron (ea)	0.5	0	0	2.5	0	1	0
Celery (ee)	0	2	1	1	5	3.5	8
Coriander (ee)	2	0	0	2	0	1	4.5
Coriander (ac)	2.5	0	0	2	0	1	4.5
Cumin (ee)	3	0	0.5	3	3	0	5
Dill (ea)	0	0	0	0	0.5	0.5	4.5
Dill (ba)	0	0.5	0	0	0.5	0	4
Fennel (ee)	2.5	1.5	1.5	1.5	4	6	5
Fennel (ac)	2	1.5	2	1.5	4	6	5
Fennel (ea)	1	2	2	1.5	4.5	6	5
Fennel (ba)	2.5	1	1.5	1.5	4.5	6	5
Jambul (wa)	0	3	0	1.5	0	3	4
Larkspur (ee)	0	1	0	0.5	1.5	1.5	1
Mustard, yellow (wa)	0	0	0	0	0	0	2
Nigella (ac)	1	8	1	1	2	1.5	9
Nux Vomica (wa)	0	0	0	0	0	0	0.5
Nux Vomica (ea)	0	0	0	0	0	0.5	0
Nux Vomica (ba)	0	0	0	0	0	0.5	0
Sabadilla (ea)	1	0	0	0	2	0	2
Sabadilla (ba)	1	0	0	0	2	0	1.5
Stavesacre (wa)	0	0	0	0	0	0	5
Sunflower (wa)	0	0	0	2	0	0	0
Tonka angostura (ee)	2	0	2.5	1.5	5	3	8
Wormwood American (ee)	4	1.5	0	1	2	1	4
Wormwood American (ea)	4	2	0	1	4	1	4

[a] Measurement from disk edge to zone edge

[b] wa—water extract, ee—ether extract, ac—acetone extract, ea—alcohol extract, ba—*n*-butyl alcohol extract

[c] Zone of inhibition absent

organisms. The extracts from 23 seeds which were found to possess greatest antimicrobial activity are listed in Table I together with the solvents used for extraction and zones of inhibition. Extracts prepared from the following seeds were found to be inactive against the seven test organisms: calabar bean, conium, fenugreek, flax, hemp, millet, mustard (black), psyllium, pumpkin (select), quince, rape, sesame, stramonium, strophanthus, watermellon, and thistle (black). The ether and acetone soluble fractions possessed the greatest antimicrobial activity. It was found that *E. coli* was most susceptible to ether, acetone, and alcohol extracts of cardamom (green) and wormwood (American), *S. aureus* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of nigella, *S. marcescens* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of tonka angostura, *M. smegmatis* to ether and acetone extracts of cardamom (green), *C. albicans* to ether and acetone extracts of celery and tonka angostura, *E. caratovora* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of fennel, and *S. Venezuelae* to ether, acetone, alcohol, and *n*-butyl alcohol extracts of nigella, celery, and tonka angostura.

It is interesting to note that the essential oils obtained from the seeds of anise, caraway, cardamom, celery, coriander, dill, fennel, and wormwood have been reported to possess antimicrobial properties with the filter paper disk method (33, 34). In this investigation extracts of these seeds were also found to possess antimicrobial properties. Whether the essential oil contained in these seeds is responsible for microbial inhibition has not been determined in this study. However, Ferenczy (22) has presented evidence to indicate that the antibacterial activity of petroselinum seeds is due to its volatile oil content.

Nine extracts of different seeds showing marked antimicrobial activity against the organisms screened in Table I were selected and concentrated. Upon screening these concentrated extracts on the seven test organisms it was found that the zones produced did not differ appreciably from the unconcentrated extracts. These concentrated extracts were further tested against 12 additional microorganisms, the results of which are found in Table II. From these data it is seen that the acetone-soluble fraction of cardamom (green) as well as the ether-soluble fractions of tonka angostura and celery possess a very wide spectrum. The activity of tonka angostura may be attributed to its coumarin content. For indeed coumarin has been reported to possess antimicrobial properties (35, 36).

TABLE II —ANTIMICROBIAL ACTIVITY OF CONCENTRATED SEED EXTRACTS

Seeds	Zone of Inhibition in mm [a]											
	S lutea	*B subtilis*	*N perflava*	*S cholerae suis*	*S typhimurium*	*P vulgaris*	*T mentagrophytes*	*M canis*	*H sativum*	*C rhodobenhani*	*P digitatum*	*E interdigitale*
Anise (ac)[b]	4	2.5	0[c]	0 5	0 5	1 5	1 5	2	4 5	1	1 5	1
Cardamon, green (ac)	8	5	3	5	4 5	8	5	3 5	8	4	5	5
Celery (ee)	7	5 5	1	1	1	5	4	6	7	3	3 5	4
Coriander (ee)	2	0 5	1	0	1	5	0	0 5	3 5	1	0 5	1
Fennel (ac)	10	1 5	0	0	0	3	14	1	5 5	5	5	10
Nigella (ac)	20	2	0	0	0	2	9	5	5	3	8	10 5
Sabadilla (ea)	9	1	0	0	0	0	2	0	0	0	2	5
Tonka angostura (ee)	8	2	1 5	2 5	1 5	1	11	1	8	9	9	13
Wormwood, American (ee)	6	1	1	3	0	2	13	2	6	7	6	14

[a] Measurement from disk edge to zone edge
[b] ac—acetone extract, ee—ether extract, ea—alcohol extract
[c] Zone of inhibition absent

SUMMARY

1 One hundred and ninety-five extracts were prepared from 39 seeds of drug origin and screened against the following test organisms· *Escherichia coli, Staphylococcus aureus, Serratia marcescens, Mycobacterium smegmatis, Candida albicans, Erwinia caratovora,* and *Streptomyces Venezuelae*

2 The solvents used for extraction were ether, acetone, alcohol, *n*-butyl alcohol, and water The ether and acetone-soluble fractions were found to have the greatest antimicrobial activity.

3 Sixteen of the seeds were found to possess no antimicrobial activity with all extracts prepared while the remaining 23 seeds were found to be active

4 Extracts of cardamom(green), tonka angostura, and celery were found to have antimicrobial activity on at least six of the seven test organisms as well as against all of the following· *Sarcina lutea, Bacillus subtilis, Neisseria perflava, Salmonella cholerae suis, Salmonella typhi murium, Proteus vulgaris, Trichophyton mentagrophytes, Microsporum canis, Helminthosporium sativum, Cryptococcus rhodobenhani, Penicillium digitatum,* and *Epidermophyton interdigitale*

5. Antimicrobial activity was not increased by concentrating the extracts

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Analysis of Barbiturate Salts in Various Dosage Forms by an Ion Exchange and Nonaqueous Titration Procedure*

By MURIEL C. VINCENT and MARTIN I. BLAKE

Barbiturate salts in various dosage forms are determined by liberating the barbituric acid from the salt by passage through a cation exchange resin. Dimethylformamide is used as the solvent. The barbituric acid is titrated visually with 0.1 *N* sodium methoxide in benzene-methanol. The method is rapid and avoids the tedious extraction procedure.

SUCCESSFUL TITRATION of barbiturates in nonaqueous solvents has been reported by numerous workers. Vespe and Fritz (1) determined barbiturates, pure and in dosage forms, by visual titration in dimethylformamide using sodium methoxide in benzene-methanol. It was noted that for tablets, the barbiturate may either be extracted prior to titration, or the powdered tablets may be titrated directly.

Pifer, Wollish, and Schmall (2) describe a procedure for the nonaqueous titration of barbiturates. Sodium salts of the barbiturates are determined by liberating the barbituric acid with sulfuric acid, followed by extraction with an organic solvent. Dimethylformamide is added and the solution is titrated with sodium methoxide.

Swartz and Foss (3) titrated barbiturates, as free acids, in a polyethylene glycol 400 and chloroform mixture. Titration was effected potentiometrically with sodium methoxide. Salts were extracted with chloroform, after acidification, using a Schmall extractor. The extracted barbituric acid was titrated by the proposed method.

Ryan, Yanowski, and Pifer (4) used lithium methoxide in benzene-methanol as titrant for barbituric acids and their salts. Salts, capsules, and tablets were extracted, after acidification, in a Schmall extractor or Squibb separator. The extracted barbiturates were titrated with lithium methoxide.

Chatten (5) titrated barbituric acids visually with potassium hydroxide in methanol, using chloroform as the solvent. It is indicated that barbiturate salts may be analyzed by liberation and extraction of the barbituric acid.

Ion exchange resins have been applied to the analysis of barbituric acids (6, 7), but not to the salts.

This report describes a procedure for the analysis of barbiturate salts, pure and in various dosage forms. A solution of the salt in dimethylformamide is passed through a cation exchange resin. The effluent, containing the barbituric acid, is titrated visually with sodium methoxide in benzene-methanol. The indicator is azo violet.

EXPERIMENTAL

Preparation of Column.—The cation exchange resin Amberlite IRC-50 was used in this study The column was prepared as reported in a previous paper (8).

Nonaqueous Titration.—All titrations were effected visually with 0.1 *N* sodium methoxide in benzene-methanol, prepared and standardized as described earlier (9). The indicator was azo violet (saturated solution of *p*-nitrobenzene-azoresorcinol in benzene).

Assay Procedure.—*Pure Barbiturate Salts.*—Approximately 100 mg. of the barbiturate salt, accurately weighed, was dissolved in 20–25 ml. of dimethylformamide, and the solution was passed through the column of resin. Additional dimethylformamide was added to the column until 50 ml. of effluent was collected. The effluent was titrated visually to the first permanent blue color. With each series of five determinations a blank was run.

With each barbiturate salt several potentiometric titrations were conducted using a Fisher titrimeter equipped with a calomel and glass electrode system. Indicator solution was added and the color change was noted at the potentiometric end point. In each case, the color change which most nearly approximated the true end point, was the first permanent blue color.

Ampuls.—Samples containing about 100 mg. of barbiturate salt were used for analysis. Ampuls containing liquids were assayed by removing a suitable aliquot and diluting to about 25 ml. with dimethylformamide. The solution was passed through the column. Fifty milliliters of effluent was collected and titrated as described for barbiturate salts. Ampuls containing solid material were analyzed by dissolving the powder in a suitable volume of dimethylformamide. An accurately measured aliquot, containing about 100 mg. of barbiturate salt, was treated in the described manner.

Capsules and Tablets.—The contents of a suitable number of capsules or tablets were dissolved in dimethylformamide. The solution was filtered into a volumetric flask, the filter was rinsed with dimethylformamide, and the flask was made up to volume. An aliquot equivalent to about 100 mg. of barbiturate salt was removed and treated in the usual manner.

* Received February 19, 1959, from the School of Pharmacy, North [illegible], Fargo
The auth[illegible] the following companies for [illegible] biturates: Abbott Laboratorie[illegible] [illegible]illy and Co., Merck Sharp and [illegible] Inc., Parke, Davis and Co., T[illegible] Laboratories

Suppositories.—A suitable number of suppositories was dissolved in dimethylformamide with the aid of gentle heat. The solution was cooled and filtered into a volumetric flask. The filter was washed with additional dimethylformamide and the flask was made up to volume. An aliquot containing about 100 mg. of barbiturate salt was analyzed as described above.

The results obtained for the recovery of barbiturate salts from various dosage forms are listed in Table I.

DISCUSSION

The successful determination of barbiturates by direct nonaqueous titration has been reported by numerous workers. It has been clearly demonstrated that the procedure is more rapid and less tedious than the official method, and is equivalent in accuracy and precision. The salts of the barbiturates, on the other hand, require an extraction procedure after conversion of the salt to the free acid, which is subsequently determined by nonaqueous titration in the usual manner. The analysis of barbiturate salts may be determined in glacial acetic acid by titration with perchloric acid, but Ryan, *et al.* (4), have indicated that the procedure is not feasible for other dosage forms because of interference due to the presence of diluents and excipients. The results of the present study indicate that a cation exchange resin can be used successfully for extraction of the free barbituric acid from the salt form by exchanging a hydrogen for the metal of the salt. The effluent is titrated with 0.1 N sodium methoxide. The method is applicable to a wide variety of dosage forms. With the exception of enteric coated tablets of pentobarbital calcium, good results were obtained as shown in Table I. No explanation can be offered for the poor precision reported for pentobarbital calcium. Application to the analysis of elixirs proved unsuccessful. A number of elixirs were studied, but consistent results were not obtained. Interfering ingredients and difficulty in removing the water from the elixir base probably contributed to the failure of the method. However, elixirs may be analyzed by employing the extraction procedure as in the official method.

Several advantages of the ion exchange modification are apparent. The method is more rapid and eliminates the tedious process of extraction. Once the column has been prepared at least 15 determinations can be effected before regeneration becomes necessary.

In the course of this investigation it was observed that blank corrections were very low, negligible in most cases. It appears that passing dimethylformamide through the resin column serves as a means of purifying the solvent. It is free of odor and acidic impurities.

TABLE I.—ANALYSIS OF BARBITURATES IN DOSAGE FORMS

Dosage Form	Recovery, %
Pure Salts	
Amobarbital sodium	99.32 ± 0.40[a] (3)[b]
Barbital sodium	100.19 ± 0.94 (4)
Butabarbital sodium	98.88 ± 0.37 (3)
Butallylonal sodium	100.92 ± 0.07 (2)
Cyclobarbital calcium	99.81 ± 0.72 (3)
Pentobarbital sodium	100.16 ± 0.21 (3)
Phenobarbital sodium	99.65 ± 0.36 (3)
Secobarbital sodium	100.71 ± 0.02 (2)
Thiopental sodium	100.22 ± 1.05 (4)
Ampuls	
Amobarbital sodium, 65 mg.[c]	100.36 ± 0.80 (3)
Hexobarbital sodium, 1 Gm.	99.47 ± 0.72 (3)
Pentobarbital sodium, 2.5 Gm.	100.11 ± 0.93 (3)
Phenobarbital sodium, 130 mg.	101.46 ± 0.77 (3)
Secobarbital sodium, 250 mg.	100.02 ± 0.61 (3)
Thiamylal sodium, 10 Gm.	99.76 ± 0.34 (3)
Vinbarbital sodium, 300 mg.	100 97 ± 0.22 (3)
Capsules	
Amobarbital sodium, 1 gr.	100.83 ± 0.83 (2)
Pentobarbital sodium, 100 mg.	99.79 ± 0.33 (3)
Secobarbital sodium, 1/2 gr.	100.73 ± 0.38 (3)
Suppositories	
Pentobarbital sodium, 200 mg.	99 36 ± 0.65 (4)
Secobarbital sodium, 1/2 gr.	100.85 ± 0.40 (3)
Tablets	
Amobarbital sodium, E.C., 200 mg.	99.75 ± 0.34 (3)
Pentobarbital calcium, E.C., 100 mg.	101.46 ± 2 79 (4)
Secobarbital sodium, E.C., 3/4 gr.	98.74 ± 0.47 (3)

[a] Based on labeled amount. [b] Number of determinations. [c] Labeled amount in unit dosage form

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A Note on the Effects of Gibberellins on Alkaloidal Content of *Hyoscyamus niger**

By JOHN Y. MASUDA and GLENN H. HAMOR

A comparative assay was made for total alkaloids of the gibberellin-treated and untreated *Hyoscyamus niger* by the U. S. P. assay procedure. It has been shown that gibberellins do not increase the alkaloidal content in the leaves The total alkaloidal content of the overground portion of the plant was somewhat less than that of the untreated plants.

THE PLANT-GROWTH stimulating properties of gibberellins, obtained from the fungus, *Fusarium moniliforme*, had been observed in Japan as early as 1926 It was not until recent years that exhaustive experimentations have been carried on in the United States

The major effects of gibberellins on plants have been reported as producing increased rate of growth and increased size and weight (1) It has also been shown that gibberellins may replace light in plants grown under low light intensity (2)

Smith and Semchetti (3) reported decrease in total alkaloids in the leaves, tops, and stems of *Datura stramonium* which were treated with gibberellic acid in concentrations of 100 and 1,000 p p m The roots, however, were observed to have increased alkaloid production They also observed decrease in total alkaloids in the leaves, tops, stems, and roots of *Atropa belladona* which were sprayed with gibberellic acid three successive times at ten day intervals In this investigation a comparative assay was conducted on treated and untreated *Hyoscyamus niger* for total alkaloidal content The plants used for this investigation were grown and obtained through the courtesy of Anton Lang of the Department of Botany, University of California, Los Angeles, and their identity certified by him

Plants of *Hyoscyamus niger*, of a biennial, purple-flowered strain, were grown from seeds in a greenhouse at a temperature of 20° in which the photo period was kept at eighteen hours by supplementing natural light with artificial illumination Crystalline gibberellin[1] was dissolved in distilled water to which a small amount of Tween 20 was added and an amount equivalent to 5 μg was applied daily with a hypodermic syringe at the base of the leaf nearest the apex of the stem (4)

The leaves showed some discoloration and seemed to be weaker in appearance than the leaves of the untreated control plant The most noticeable effect of of the gibberellin-treated plant was stem elongation, with resultant increase in the internodes There was considerable increase in total dry weight in the treated plants due to increase in stem weight, but the weight of the leaves was shown to decrease slightly The data on weight, height, and alkaloidal content are given in Table I The figures shown are averages of 24 plants and the weights given are average weight per plant

TABLE I

Age, Days	Dry Weight, Gm	Alkaloids, % Sample 1	Sample 2	Sample 3	Height, Inches
		Treated Plants			
21	2 3				14 5
	Leaves, 1 12	0 171	0 166	0 168	
	Stem, 1 18	0 023	0 023	0 022	
28	2 55				21 5
	Leaves, 1 14	0 128	0 130	0 129	
	Stem, 1 41	0 014	0 016	0 017	
35	2 85				26 0
	Leaves, 1 13	0 127	0 126	0 129	
	Stem, 1 72	0 014	0 013	0 013	
42	3 14				36 5
	Leaves, 1 09	0 123	0 124	0 122	
	Stem, 2 05	0 005	0 006	0 006	
		Untreated Plants			
	Leaves				
21	1 23	0 169	0 178	0 171	6 0
28	1 24	0 142	0 146	0 140	6 1
35	1 26	0 144	0 142	0 139	6 2
42	1 29	0 153	0 150	0 158	6 5

The U S P assay procedure was used but, because of the limited amount of sample, the weight of the samples used was one-half the required weight The plants were harvested at intervals of seven days and were immediately dried for two days in a circulating oven at a temperature of 40° This material was ground and analysis performed immediately

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* Received June 21, 1958 from the School of Pharmacy, University of Southern California Los Angeles

[1] Supplied by Eli Lilly and Co Indianapolis Ind, containing gibberellin A_3 and up to 10% of unknown but apparently inactive materials

Communication to the Editor

The Amount of Fines Necessary for a Tablet Granulation

Dear Sir:

Numerous articles, books, and advertising pieces discussing compressed tablet manufacture mention the importance of "fines." Occasionally, 10–20% is set as the maximum amount of "fines" permissible. It is interesting to note that "fines" are rarely defined.

We are of the opinion that there is no general limit to the amount of "fines" (whatever they may be) that can be present in a granulation. Objections to "fines" must be determined for each specific formula.

To illustrate that "fines," in the freely used sense, are not always objectionable, the following examples are listed.

A. Tablets of 3-phenylpropylcarbamate, 400 mg. each, were prepared. Sieve analysis of the granulation indicated that all material passed through a No. 30 screen and 50% through a No. 200 screen. The tablet was successfully compressed at approximately 300 tablets per minute on the Stokes Model B-2 rotary tablet machine.

B. A standard APC tablet using 40 mesh aspirin crystals and spray dried acetophenetidin (1) was prepared by direct compression. Sieve analysis indicated that all the material passed through a No. 30 screen, 84% through a No. 60 screen, and 62% passed through a No. 100 screen. The tablets were successfully compressed at approximately 1,400 tablets per minute on a Stokes Model BB-2 rotary tablet machine.

C. A placebo spray-dried calcium sulfate granulation was prepared. Sieve analysis indicated that 92% passed through a No. 100 screen. The tablet was successfully compressed at approximately 700 tablets per minute on a Stokes Model B-2 rotary tablet machine.

D. A standard wet-granulated calcium sulfate granulation was prepared. Sieve analysis indicated that all the material passed through a No. 30 screen and 84% through a No. 100 screen. The tablets were successfully compressed at approximately 2,000 tablets per minute on a Stokes Model BB-2 rotary tablet machine.

E. Tablets of chlorpromazine, 25 mg. each, were prepared by a standard wet granulating technique. Sieve analysis indicated that 20% of the material was retained on a No. 20 mesh screen, 40% retained on a No. 100 mesh screen, and 40% passed through a No. 100 mesh screen. The tablets were successfully compressed at approximately 1,800 tablets per minute on a Stokes Model BB-2 rotary tablet machine.

We do not wish to imply that "fines" are not an integral part of a granulation, nor do we wish to imply that they do not influence compression. For each specific granulation one must determine what portion (of the smaller mesh size) is unacceptable, and why it is unacceptable; this then could be termed excessive "fines."

(1) U S pat. 2,798,838, July 9, 1957.

Smith Kline and French Laboratories
Philadelphia, Pa.

STANLEY J. TUCKER
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Received April 14, 1959.

Book Notices

The Chemistry of Drugs. 3rd ed. By NORMAN EVERS and DENNIS CALDWELL. Interscience Publishers, Inc., New York, 1959. 415 pp. 15 x 23 cm. Price $12.25.

This is an entirely new book, the third edition published in 1933 having been completely revised. The new third edition deals with the chemistry of both synthetic and natural drugs, their structure, methods of preparation, and synthesis, and in addition, their chemical properties and therapeutic uses. Comprehensive tables of official or approved names of drugs together with their proprietary names and chemical names are also included. Approximately half of the book is devoted to synthetic drugs, and the other half to naturally occurring medicinal chemicals.

Textbook of Physiology and Biochemistry. By GEORGE H. BELL, J. NORMAN DAVIDSON, and HAROLD SCARBOROUGH. The Williams and Wilkins Co., Baltimore, 1959. xi + 1,065 pp. 16.5 x 24 cm. Price $12.50.

This is the fourth edition of a book that is intended to be an introduction to the study of physiology and biochemistry. In order to bring the text up to date, considerable rewriting has been done, even though the third edition was published only two years ago. The book is intended primarily for medical students, but should be useful to students in related fields. Numerous cross references correlate the information presented under different sections of the subject. References are given after each of the 55 chapters, and an index is appended.

Recent Studies in Yeast and Their Significance in Industry. S. C. I. Monograph No. 3. The Macmillan Co., New York, 1959. 162 pp. 14 x 21.5 cm. Price $2.95.

This book includes papers and discussions presented during a Symposium organized by the Dublin and District Section of the Microbiology Group and the Food Group held in Dublin in September 1956. Six sessions were held during the Symposium, during which recent advances in research on yeasts were presented.

Mono- and Sesquiterpenoids. Vol. II of *The Chemistry of Natural Products.* By P. DE MAYO. Interscience Publishers, Inc., New York, 1959. vii + 320 pp. 15 x 23 cm. Price $7.50.

The Higher Terpenoids. Vol. III of *The Chemistry of Natural Products.* By P. DE MAYO. Interscience Publishers, New York, 1959. vii + 239 pp. 15 x 23 cm. Price $6.

The first volume of this important series on the chemistry of the alkaloids was described briefly in THIS JOURNAL, 47, 232(1958). Volumes II and III, dealing with the chemistry of the mono- and sesquiterpenoids of the higher terpenoids, respectively, have recently been published simultaneously. Both volumes represent an important contribution to the chemistry of these important and interesting compounds. The terpenoids provide a clear concept of the versatility of carbonium ion rearrangements and furnish a useful framework upon which many general chemical reactions may be studied. These include synthetic and degradative processes and furnish examples of the sterochemical requirements of reactions and of the relationship between sterochemistry and stability and other physical properties.

Methoden der Organischen Chemie (Houben-Weyl). Stickstoff-Verbindungen II/III. 4th ed. Band XI/2 Edited by EUGEN MÜLLER. Georg Thieme Verlag, Stuttgart, 1959. xlviii + 840 pp. 17 x 25.5 cm. Price DM 155, subscription DM 139.50.

This volume represents the tenth in a series of a completely new and revised fourth edition of "Methoden der Organischen Chemie (Houben-Weyl)," the first of which was published in 1952. The publication of the volumes have not run consecutively, and it is expected that at least fourteen will be required to complete the new revision. The first in the series was Band VIII, covering the third part of oxygen-containing compounds. The present volume, XI/2 contains a conclusion of the second part of XI/1 which was devoted to Nitrogen Compounds II. Band XI/1 was described in THIS JOURNAL, 47, 612(1958). About one-third of Band XI/2 covers the transformation reactions of primary and secondary amines. Conversion reactions by which amines are transformed into other substances such as acid amides, amidines, nitramines, and hydroxamines are comprehensively presented, and deamination reactions are discussed. In addition, special acylation methods are competently discussed for the introduction of acid radicals such as formyl, acetyl, and benzoyl into amines.

Nitrogen Compounds III is composed of five chapters. In the first chapter, which is relatively short, the preparation and transformations of 1,2- and 1,3-alkylenimines are discussed. The second, and one of the largest chapters is devoted to methods for the preparation and transformations of amino acids and their derivatives. This chapter is particularly outstanding because of its completeness of treatment of the important group including amino acids. Analytical methods for these compounds are adequately covered.

The fourth chapter, which also is quite extensive, covers preparative methods and other pertinent information relating to quaternary ammonium compounds. The fifth and last chapter deals with nitrogen-sulfur compounds and follows the usual arrangement of the other chapters by providing a general introduction to the subject and information concerning methods of synthesis and transformation reactions. Volume XI/2 contains an author and a subject index, both of which are well designed and which enable the reader to locate information quickly.

Colorimetric Determination of Traces of Metals. 3rd ed. By E. B. SANDELL. Interscience Publishers, Inc., New York, 1959. xxii + 1,032 pp. 15 x 23 cm. Price $24.

The general plan of treatment of the subject in this third edition is the same as in the second edition, which was reviewed in This JOURNAL, 40, 177(1951). Methods that have been published since 1950 were reviewed. Some have replaced former methods, and other older methods have been revised. The many references and the indexes enhance the value of this book to the analytical chemist.

Modern Pharmacognosy. By EGIL RAMSTAD. Blakiston Division, McGraw-Hill Book Co., Inc., New York, 1959. viii + 480 pp. 15 x 23 cm. Price $10.50.

This textbook presents drugs of biological origin with emphasis on their biochemical relationships The drugs are arranged in chapters according to chemical classifications. The latter part of the book takes up commercial drug production, formation of drug constituents, variability in drug activity, preservation and storage, and analysis of natural drug products. An index is appended.

Cell and Tissue Culture. By JOHN PAUL. E. & S. Livingstone Ltd., Edinburgh and London, 1959 Distributed in the U. S. by the Williams & Wilkins Co., Baltimore. viii + 261 pp. 14 x 21.5 cm. Price $7.

An up-to-date account of the techniques and applications of the important subject of tissue culture is presented. The text is based on the instruction given at the Tissue Culture Association Summer Course, and has been written particularly with the needs of participants of that course in mind. The book is divided into four parts. In Part I, the principles of cell culture are outlined in a series of six chapters. This is followed by comparable treatment of subjects pertaining to the preparation of materials, special techniques, and applications of cell and tissue culture methods.

American Drug Index 1959 By CHARLES O WILSON and TONY EVERETT JONES J B Lippincott Co, Philadelphia, 1959 671 pp 13 5 x 20 5 cm Price $5 75

This is the fourth annual edition of a useful compilation of generic, trade, and chemical names and composition of drugs and dosage forms with indicated uses Although the text has been brought as up to date as possible with the addition of new drugs, the elimination of less useful material has enabled the reduction from 716 pages (1958) to 671 pages

New and Nonofficial Drugs 1959 Evaluated by the A M A Council on Drugs J B Lippincott Co, Philadelphia, 1959 xxvii + 687 pp 12 x 19 cm Price $3 35

The 1959 edition of this annual publication of the Council on Drugs of the American Medical Association contains descriptions of drugs evaluated on the basis of available data and reports of investigations Monographs presented under nonproprietary names are designed to provide chemical or biologic identity, including pertinent properties, actions and uses, including associated side effects, toxicity, and precautions, dosage, including routes of administration, available preparations, and applicable commercial names The value of this book to the medical and related professions makes each volume a necessary addition to all libraries

The Effect of Pharmacologic Agents on the Nervous System Vol XXXVII Edited by FRANCIS J BRACELAND The Williams & Wilkins Co, Baltimore, 1959 xi + 488 pp 15 5 x 23 cm Price $13 50

The Association for Research in Nervous and Mental Disease held a symposium in 1957 on "The Effect of Pharmacologic Agents on the Nervous System" This book constitutes the proceedings of the symposium and contains reports by outstanding authorities on clinical experience, evaluations, and aspects of research activity It also includes reviews of the literature

Antibiotics Annual 1958–1959 Edited by HENRY WELCH and FELIX MARTI IBAÑEZ Medical Encyclopedia, Inc, New York, 1959 xvii + 1,107 pp 17 x 25 cm Price $12

This annual publication reports the Proceedings of the Sixth Annual Symposium on Antibiotics which was held in Washington, D C, October 15, 16, and 17, 1958 These dates coincided with the thirtieth anniversary of the discovery of penicillin in London's St Mary's Hospital It was, therefore, appropriate that the first session of the Symposium was devoted to a series of papers on the history and development of antibiotics During other sessions, a large number of papers on recent advances in the field of antibiotics was presented Altogether more than 175 papers are included in the 1958–1959 Antibiotics Annual The publication of this series on antibiotics constitutes an outstanding contribution and an important and convenient source of information of value to research workers and to others interested in the fascinating subject of antibiotics

Cahiers de Synthèse Organique Vol V By JEAN MATHIEU and ANDRÉ ALLAIS Masson et Cie, Paris, 1959 394 pp 15 x 22 cm Price paper bound, 7,800 Francs, cloth, 8,500 Francs

Volume V of this series (in French) is concerned with molecular degradations The first section (numbered 12) deals with the loss of a functional carbon The second section (numbered 13) takes up the cleavage of carbon chains and the rupture of carbocycles The style of presentation is similar to that in Vol IV which was described in THIS JOURNAL, 47, 836(1958)

Staphylococcus Pyogenes and Its Relation to Disease By STEPHEN D ELEK E S Livingstone Ltd, Edinburgh and London, 1959 Exclusive U S Agents, The Williams & Wilkins Co, Baltimore vii + 767 pp 15 x 24 cm Price $15

This book is a monograph on *Staphylococcus pyogenes* and its relation to disease This relation has been emphasized dramatically by the antibiotic resistant infections due to this microorganism in many hospitals The text includes chapters on problems of taxonomy, bacteriological techniques, metabolism, serological studies, virulence and disease, action of combatting agents, and clinical problems A bibliography of publications covers 188 pages Subject and author indexes are appended

Six-Membered Heterocyclic Nitrogen Compounds With Three Condensed Rings By C F H ALLEN Interscience Publishers, Inc, New York, 1958 xxii + 624 pp 15 x 23 cm Price $26 single, $24 subscription

This is the twelfth volume of a series of monographs devoted to a modern, detailed, and comprehensive presentation of heterocyclic chemistry, each prepared by a group of authorities The subdivisions have been designed to cover the field in its entirety by monographs which reflect the importance of the interactions of the various compounds

This volume covers polynuclear heterocyclic compounds containing nitrogen and three fused six-membered rings These compounds are not only of unusual theoretical interest, but of considerable practical importance to the drug industry The arrangement and the discussion of the various classes emphasize their similarity and significant differences The literature has been covered through 1952 The book is divided into nine general sections, the subject matter of which is arranged as follows I Azaanthracenes, II Azaphenanthrenes, III 4 Azaphenanthrenes, IV 5 Azaphenanthrenes, V Diazaphenanthrenes (except Phenanthrolines), VI 1,10 ,1,7 , and 4,7- Diazaphenanthrenes, VII Other polyazaphenanthrenes, VIII Azabenzonaphthenes, IX The ultraviolet absorption spectra of polycyclic heterocyclic aromatic compounds

The "a" system of nomenclature has been used as in earlier volumes All individual chemical substances, however, are also named according to the system employed by *Chemical Abstracts*, and the index includes all names

The editor and the contributors seem to have accomplished their aim in writing this treatise, and the subject has been treated in a most competent and scholarly manner

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII | *JULY 1959* | NUMBER 7

Some Aspects of the Pharmacology of Ethyl-4-phenyl-1-[2-(phenylamino)ethyl]-piperidine-4-carboxylate (Win 13,797)*

By CHARLES BERRY†, ALAN BURKHALTER‡, LESTER W. DAVIS, E. G. GROSS, and H. H. KEASLING

The pharmacological activity of an analog of meperidine has been compared with morphine and meperidine and its effects evaluated when tested for toxicity, on toothpulp thresholds, respiration, the gastrointestinal tract, and circulation. It is concluded that Win 13,797 is much more active than morphine on a milligram basis but otherwise is qualitatively similar.

THE MANY side effects of presently available potent analgetics have prompted the continuing search for new agents. The substitution of phenylamino ethyl for methyl on the nitrogen atom of meperidine yields a material which seemed to have promise as an analgetic. This report details a pharmacologic survey of the activity of this compound: ethyl-4-phenyl-1-[2-(phenylamino)ethyl]-piperidine-4-carboxylate (Win 13,797), the structure of which is shown in Fig. 1

EXPERIMENTAL

Acute Toxicity.—The intravenous LD_{50} was determined in mice weighing about 30 Gm. Groups of 10 mice were utilized at each dose The LD_{50} and the 95% confidence limits were calculated by the method of probits as described by Finney (1).

Toothpulp Thresholds.—The elevation of thresholds following electrical stimulation of the toothpulp in rabbits receiving doses of morphine or Win 13,797-3 was measured by the method of Yim, *et al.* (2)

Respiration.—(*a*) Minute volumes were determined simultaneously during the experiments on toothpulp thresholds as previously described by Yim (2)

(*b*) pCO_2 Sampling: Rabbits were anesthetized with ether and polyethylene cannulas were inserted into the trachea and tied firmly in place. The skin incision was closed with wound clips and the animals were allowed to recover for at least four hours. The pCO_2 of the expired air was sampled via a polyethylene catheter leading to an LB-1 infrared CO_2 gas analyzer. When stable control levels were obtained drugs were administered subcutaneously and the end-expired pCO_2 sampled at intervals of fifteen, thirty, forty-five, and sixty minutes.

(*c*) Placental transmission: Pregnant rabbits were anesthetized by the spinal administration of lidocaine hydrochloride (1%) one or two days before term. A laparotomy was performed and the uterus exposed. In a modification of the technique of Halasey and Dille (3) one or two fetuses were removed to serve as controls and intravenous drug administered to the mother. Five minutes later the remaining fetuses were delivered and respiratory rate counts were made at five-minute intervals.

Gastrointestinal Tract.—(*a*) The inhibition of the passage of a charcoal meal was determined by the technique of Macht and Barba-Gose (4). Four young, mature rats were used at each dose.

(*b*) The activity of the stomach and intestine of the awake dog was recorded by means of water-filled balloons inserted in a stomach fistula and a Thiry-Vella loop. The dogs were trained to lie quietly upon a table during the process of the recording and, following a control period, the drugs were administered subcutaneously.

* Received April 8, 1959, from the College of Medicine, State University of Iowa, Iowa City

Presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Ann Arbor, Mich., August, 1958

This paper is based on a thesis submitted to the Graduate College of The State University of Iowa by C Berry in partial fulfillment of the requirements for the degree of Master of Science

Win 13,797 was obtained from Sterling-Winthrop Research Institute, Rensselaer, N Y

† Public Health Service Research Fellow of the National Institutes of Health

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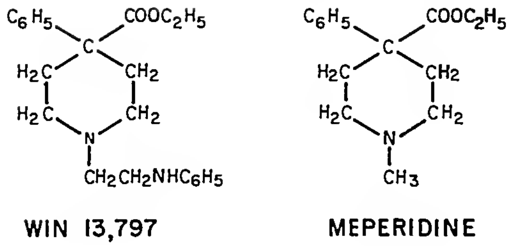

Figure 1.

(*c*) T-tubes were placed in the gall bladder of dogs anesthetized with ether A reservoir of normal saline was arranged to supply a constant rate of perfusion of saline into the gall bladder. Recordings were made through a U-shaped manometer containing a float inserted into the perfusion circuit as described by Butsch, *et al.* (5). Forty minutes after the ether was discontinued control tracings were obtained and drugs were administered subcutaneously.

Circulation.—The arterial pressure of unanesthetized rabbits was recorded from the femoral artery. The artery was catheterized utilizing infiltration anesthesia and connected to a Statham transducer. The transducer output was amplified and recorded with an Offner Dynagraph (Type 506). Drugs were administered intravenously. The same recording system was utilized in dogs anesthetized with a mixture of 15 mg./Kg. of thiopental sodium and 250 mg./Kg of barbital sodium.

Drugs.—Fresh drug solutions were prepared in 0 9% saline for each experiment. The materials utilized were Win 13,797-3,[1] morphine sulfate, meperidine hydrochloride, nalorphine hydrochloride, epinephrine chloride, levarterenol bitartrate, and acetylcholine chloride

RESULTS AND DISCUSSION

The appearance of animals receiving Win 13,797-3 is qualitatively similar to that of animals treated with morphine. With sublethal doses of morphine sulfate or Win 13,797-3 in mice the Straub tail phenomena was characteristic. Following larger doses of morphine, mice exhibit a short excitement stage followed by clonic and tonic convulsions from which death may ensue. Following larger doses of Win 13,797-3 the same series of effects may be observed although the onset of effect may be more rapid In qualitative studies in rabbits high doses of Win 13,797-3 produced convulsions which were indistinguishable from those induced by morphine. The intravenous LD_{50} in mice of morphine was determined to be 180 mg./Kg. with 95% confidence limits of 90–210 mg./Kg. while the LD_{50} of Win 13,797-3 was 22 mg./Kg. with 95% confidence limits of 20–25 mg./Kg.

Toothpulp thresholds and respiratory minute volumes were determined by administering Win 13,797-3 or morphine subcutaneously to rabbits. Changes were expressed as percentages of the control values. Figure 2 depicts a time-response curve for Win 13,797-3 at doses of 1 and 4 μM./Kg. for a time period of one hundred and fifty minutes. The upper

[1] The material utilized exclusively in these experiments was the ethane sulfonate salt having a molecular weight of 462 6, identified as Win 13,797-3. The number Win 13,797 refers to the base.

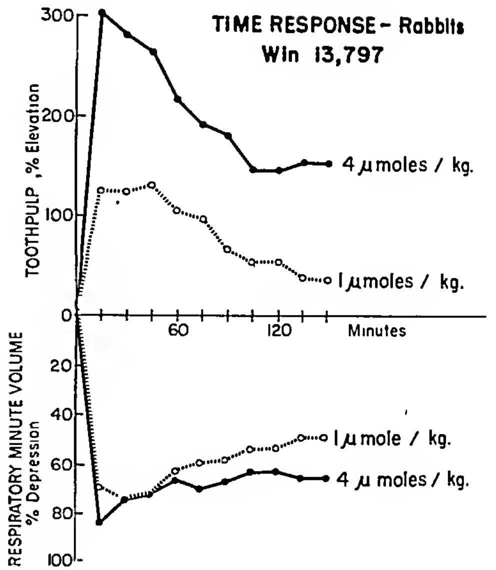

Figure 2.

curves represent percentage elevations in toothpulp thresholds while the lower curves represent percentage depression of respiratory minute volumes. It will be noted that a peak of effect is seen fifteen to thirty minutes after drug administration. In contrast, morphine sulfate peak effects are noted forty-five to sixty minutes after drug administration. In other respects the time-response curves for morphine and Win 13,797-3 at equieffective doses are essentially similar.

The relative potency of Win 13,797-3 and morphine on a molar basis was determined using toothpulp thresholds in a 4-point parallel line assay after the method described by Finney (1). With regard to the increase in toothpulp threshold Win 13,797-3 is 19 times as active as morphine. The fiducial limits at the 95% level are 14 and 27.

Recent investigations in our laboratory have elucidated the oral efficacy of morphine upon the rabbit toothpulp threshold. The ratio of effectiveness was compared using the 4-point parallel line assay of Finney (1). Administration of subcutaneous morphine was 9 times as effective as orally administered morphine with fiducial limits at the 95% level of 6–13. Attempts to compare the oral effectiveness of Win 13,797-3 with the subcutaneous doses showed that even at oral doses 100 times as high as the hypodermic dose the activity of the oral dose was substantially lower. Further consideration of this approach did not appear fruitful.

The antagonism of Win 13,797-3 by nalorphine was studied utilizing simultaneous subcutaneous administration. Mixtures of equal amounts, $^1/_3$, $^1/_{10}$, and $^1/_{30}$ the molar quantities of nalorphine with a fixed 2 μM./Kg. dose of Win 13,797-3 were compared. Antagonism of toothpulp threshold elevation in all ratios is significant at the 0.05 level. Respiratory minute volume depression was significantly antagonized only by the 1:1 and 3:1 ratios.

Numerous objections have been raised to the use of respiratory minute volume data as a measure of respiratory depression in man, Seed, *et al.* (6). In a series of exploratory experiments numerous attempts

to adapt the rebreathing technique described by these authors to the rabbit have not revealed a consistent correlation between inspired pCO_2 and alveolar ventilation. These studies are continuing in the hope of resolving this problem. Some evidence of respiratory depression was gained, however, by measuring the end-expired pCO_2. Control levels were quite variable between rabbits. While the tracings indicated that end-expired samples were being obtained it was noted that these levels varied quite dramatically with rate in control studies within the same animal. Both Win 13,797-3 and morphine, in doses producing equivalent toothpulp threshold increases, induced qualitatively similar depression of rate and increase in pCO_2 levels. The administration of intravenous nalorphine produced a prompt reversal of depression as evidenced by return of respiratory rate and pCO_2 levels to control values, in contrast to the findings reported by Lasagna (7) and Keats (8) in man.

As with morphine sulfate or meperidine hydrochloride, Win 13,797-3 is capable of passing the placental barrier and inducing respiratory rate depression in the fetus. Pregnant rabbits just before term were given, intravenously, either morphine sulfate, 4 μm./Kg.; meperidine hydrochloride, 14 μM./Kg.; or Win 13,797-3, 1 μM./Kg. Five to ten minutes after drug the respiratory rates of the drugged fetuses, when compared to the control fetuses, had decreased from $^1/_3$ to $^1/_2$. This depression was rapidly reversed by subcutaneous administration of 0.25 mg. of nalorphine to each of the drugged fetuses.

The effects of Win 13,797-3 and meperidine hydrochloride on the intestinal tract were compared by their ability to inhibit the passage of a charcoal meal in rats. The doses and per cent inhibition resulting from intraperitoneal injection of meperidine hydrochloride were: 80 μM./Kg.–6% and 160 μM./Kg.–62%. For Win 13,797-3 the values were: 1 μM./Kg.–9% and 2 μM./Kg.–56%.

Qualitative gastrointestinal studies were also made in awake dogs in which Thiry-Vella loops and stomach fistulas had been previously prepared. The dogs were utilized only two days a week and subcutaneous doses of morphine sulfate, meperidine hydrochloride, and Win 13,797-3 were used. In all instances the results observed were qualitatively similar. Shortly after drug administration a prolonged rise in intestinal tone was observed accompanied by relaxation and decreased motility of the stomach. This phenomenon was usually preceded, in the case of morphine and Win 13,797-3, by spasms of the stomach musculature and the dogs were observed to retch and lick their chops periodically.

When intrabiliary pressure was measured in dogs whose gall bladder was perfused with a constant rate of flow of saline the subcutaneous administration of Win 13,797-3 resulted in an increase of pressure qualitatively similar to that observed when the dogs were injected with morphine.

Qualitative comparisons of the cardiovascular effects of Win 13,797-3 and morphine in rabbits and dogs did not reveal any significant differences between the compounds. The intravenous administration of either drug resulted in a rapid and prolonged depressor response of the blood pressure which was slow to recover. Intravenous titration with nalorphine rapidly reversed this depressor response. Blood pressure responses of small doses of epinephrine, levarterenol, and acetylcholine measured before and after the administration of Win 13,797-3 or of morphine did not differ markedly between the two compounds. Quantitative comparisons were not possible due to the confounding of the responses imposed by the prolonged depression of the blood pressure.

Tracings of the blood pressure of normal awake rabbits showed that there was immediate reflex compensation of the blood pressure when the animals were tilted at a 45° angle from the horizontal. After the subcutaneous administration of morphine or of Win 13,797-3 the blood pressure response to tilting of the rabbit consisted of a transient pressor overshoot before reflex compensation occurred.

SUMMARY

Comparisons have been made concerning the pharmacological influence of morphine, meperidine, and Win 13,797-3. No qualitative differences were observed to exist between the compounds. Although, on the basis of toothpulp threshold comparisons, Win 13,797-3 is 19 times as effective in molar concentrations as morphine, it seems to possess no advantages over presently known analgetic compounds.

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The Evaluation of Iodinated Organic Compounds as Radiopaque Media*

By JAMES O. HOPPE†

The general properties of X-ray contrast media are discussed with particular reference to the use of iodinated organic compounds in cholecystography and urography. The development of test procedures for the evaluation of radiopaques for X-ray visualization of the gallbladder and the upper urinary tract is described. Relative oral cholecystographic activity, from most to least active, was observed in the cat as follows: iopanoic acid, iophenoxic acid, iodoalphionic acid, and iodophthalein, in agreement with the relative order of activity reported in the literature for these compounds in man. Diatrizoate sodium was found to produce earlier and better X-ray visualization of the upper urinary tract in the rabbit with less than half the systemic toxicity observed with iodopyracet. The acute intravenous toxicity of diatrizoate sodium in the mouse, rat, rabbit, cat, and dog was found to be approximately half as great as that observed for iodopyracet, sodium iodomethamate, or sodium acetrizoate. An iodinated aromatic nucleus, to provide radiopacity, was found to be common to both cholecystographic and urographic media. The cholecystographic medium required a "transport mechanism" consisting of an alkanoic acid side chain containing 6 to 7 carbons with 2 iodines in the aromatic nucleus and 5 carbons with 3 iodines in the radiopaque portion of the molecule. The urographic contrast medium required a carboxyl group to provide high water solubility and could contain an alkanoic acid chain with no more than three carbons.

THE WORD RADIOPAQUE is used as an adjective to denote opacity to radiant energy and as a noun, synonymously with the term X-ray contrast medium, to identify specifically those substances which inhibit the passage of Roentgen or X-rays. The property of radiopacity, inherent in a variety of chemical elements, has increased immensely the boundaries of the science of diagnostic radiology. It was apparent almost from the moment of Roentgen's discovery of the X-ray in 1895 (1) that here was an important new medical tool which was capable of producing an image on photographic film corresponding to the morphology of the bony tissues but not of the soft tissues of the body. Radiopaque media have made it possible for physicians to examine the anatomy and observe the physiology of the stomach, gallbladder, urinary tract, heart, blood vessels, lungs, and other soft tissue structures without having to resort to exploratory surgery. These substances are important diagnostic aids to the radiologist in enabling him to establish the presence or absence of disease in various organs within the body. It is the purpose of the present communication to discuss briefly the general properties of radiopaques and, in particular, the development of test methods for evaluating iodinated organic compounds as cholecystographic and urographic media.

* Received May 4, 1959, from the Pharmacology Section, Sterling Winthrop Research Institute, Rensselaer, N. Y.
Presented to the Scientific Section of the A.PH.A., New York meeting, May 3, 1957.

† Recipient of the Ninth (1957) Chilean Iodine Educational Bureau Award in recognition of his development of quantitative laboratory methods for evaluating iodine containing diagnostic drugs and his many other contributions to the study of iodine containing compounds.

GENERAL PROPERTIES OF RADIOPAQUES

Function of a Radiopaque.—The use of a radiopaque might be illustrated with the aid of the two X-ray pictures reproduced in Figs. 1A and 1B. Figure 1A shows an X-ray view of the upper right quadrant of the human abdomen. The ribs, lumbar vertebrae, and crest of the ilium are clearly visible. Also present in this same area are the gallbladder, right kidney, blood vessels, and portions of the stomach, liver, and intestines, but these structures cannot be seen. Figure 1B shows an X-ray view of the same area in the same patient some twelve hours after the oral ingestion of an iodinated organic compound specifically designed to localize in the bile in the gallbladder. The presence of the radiopaque (iopanoic acid, U. S. P., Telepaque[1]) in the bile prevented the passage of the X-rays during the exposure to leave an image on the film corresponding to the morphology of the normal human gallbladder which appears as a white, pear-shaped mass near the center of Fig. 1B.

Physical Properties.—One might ask why the bony tissue was visible whereas the soft tissue structures were not. Further, why did the radiopaque cause the gallbladder to become visible in Fig. 1B? Since the image on the X-ray film depends upon the amount of X-rays which pass through the subject, the answers to both questions can be found with the aid of the data in Table I.

The formula states that the ability of an element to absorb X-rays (μ) varies according to the third power of the wavelength (λ) and the fourth power of the atomic number (Z). The value "k" is a constant and the value "0.2" is a factor to account for the scattering effect depending upon the wavelength at which the measurements are made. It is readily apparent that the absorption of X-rays increases enormously as the atomic number goes up. The soft tissue structures of the body, composed of carbon, hydrogen, oxygen, and nitrogen with low atomic

[1] Product of Winthrop Laboratories, New York, N. Y.

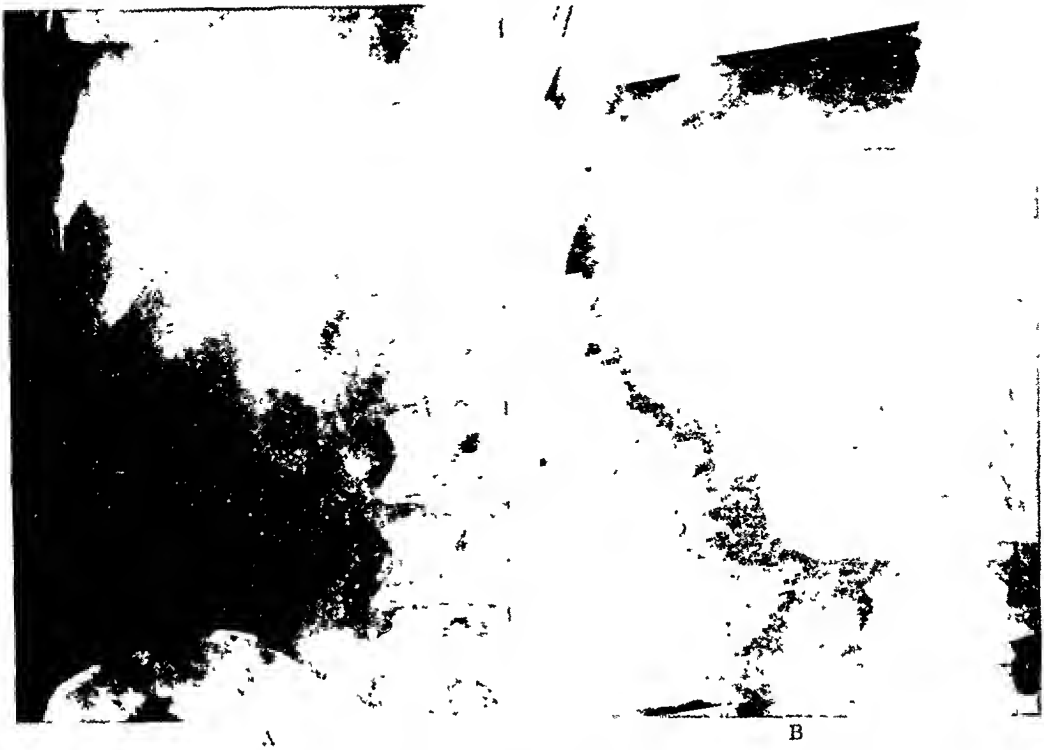


Fig 1 —Cholecystography in man. A. X-Ray picture before cholecystographic contrast medium B X-ray picture at twelve hours after oral ingestion of iopanoic acid (X-ray pictures furnished by Dr. William Shehadi, New York Polyclinic Hospital, New York, N Y)

TABLE I —ABSORPTION OF X-RAYS IN RELATION TO ATOMIC NUMBER

$$\mu = k\lambda^3 Z^4 + 0.2 \qquad (2)$$

Element	Atomic Number
Hydrogen	1
Carbon	6
Nitrogen	7
Oxygen	8
Phosphorus	15
Calcium	20
Bromine	35
Iodine	53
Barium	56
Mercury	80
Lead	82
Thorium	90

numbers, appear on the X-ray film as an undifferentiated mixture of black and gray shadows In contrast, the bony tissues, composed of calcium and phosphorus with considerably higher atomic numbers, appear as sharply defined white areas on the X-ray film

It would appear, therefore, that the ideal contrast medium would incorporate an element with a very high atomic number Proceeding down the list of elements in Table I to thorium, with an atomic number of 90, one might assume that this element would make an excellent X-ray contrast medium Indeed, it was used in the form of a thorium dioxide sol for visualizing blood vessels but was later found to possess two serious disadvantages: (*a*) it is retained indefinitely in the body after intravenous injection and (*b*) thorium is radioactive (3, 4) Even more disconcerting, thorium has a half-life of 1.34×10^{10} years (5) Lead and mercury have defied the efforts of the chemist to synthesize acceptable molecules containing these elements to absorb X-rays They do not form a stable carbon bond and, hence, are not safe from the hazards of heavy metal poisoning when used in soluble form in the large dosages necessary for X-ray contrast visualization Barium, in the form of insoluble barium sulfate, is one of the oldest useful radiopaque elements used for gastrointestinal visualization but possesses the same disadvantages of lead and mercury in the soluble form The two halogens, bromine and iodine, both form firm carbon bonds and can be incorporated into a wide variety of organic structures permitting high water solubility and low toxicity Although it possesses a lower atomic number, bromine absorbs X-rays more effectively than iodine in the longer wavelength of the X-ray spectrum (0.4 to approximately 1.0 Å) Iodine, on the other hand, absorbs X-rays much more effectively than bromine in the shorter wavelength (0.2 to approximately 0.4 Å) The long wave length X-rays are not useful in producing an X-ray image of soft tissue structures because of lack of penetration and the greater hazard of radiation injury This, then, narrows the field down to the element iodine at the present time Iodine is the most widely used element in the designing of radiopaques for two important reasons: (*a*) it will form a firm carbon bond and (*b*) this property enables the chem-

ist to incorporate iodine into many different types of organic structures so that it can be administered safely in the large quantities necessary for the X-ray visualization of soft tissue structures

Biological Properties —From these considerations, it is possible to set forth the essential biological properties which a compound must possess in order to be acceptable as a radiopaque

High Radiopacity —Radiopacity is an obvious requirement in order to classify the compound for pharmacological evaluation High radiopacity is listed under biological properties because the greater the ability of a compound to inhibit the passage of X-rays, the less of the material will be required, thus providing greater specificity of action

Low Systemic and Local Tissue Toxicity —Low toxicity, both at the systemic and local tissue levels, is extremely important because of the massive dosages used and the conditions under which these doses are administered For example, in visualizing the chambers of the heart, a volume of 50 cc of a 50 to 90% aqueous solution may be injected intravenously in two seconds or less

Pharmacodynamically Inert —In most instances the patients who receive compounds of this type are not in the best state of health The radiopaque should not aggravate their condition by causing hypotension, vomiting, nausea, dizziness, diarrhea, headache, etc The critical demands upon a radiopaque compound are contrary to the usual pursuit of the pharmacologist who looks for ways and means of increasing pharmacological activity In the case of the radiopaque, it is essential that it be pharmacodynamically inert

Selective Localization —The gastrointestinal tract can be readily visualized by swallowing a barium sulfate meal It is necessary to resort to indirect methods in order to visualize the less readily accessible structures as the gallbladder or the upper urinary tract The radiopaque should be capable of being transported in the blood to the specific organ in question and there concentrated in the normal tissue fluid to cause the soft tissue structures to visualize on X-ray film

Prompt and Complete Elimination —Once the X-ray pictures have been taken, there is no longer any need for the presence of the radiopaque and, therefore, it should be promptly and completely eliminated with no discomfort to the patient

CHOLECYSTOGRAPHY

Cholecystography is the roentgenographic examination of the gallbladder with the aid of an X ray contrast medium Calcified stones in a gallbladder would be visible in the ordinary X ray picture as in Fig 1A without the aid of an X ray contrast medium The great majority of gallstones, however, are composed of cholesterol or a mixture of cholesterol and bile pigments The elemental composition of these stones is similar to that of the tissues of the gallbladder itself and, consequently, are not visible in the ordinary X ray picture Gall stones of this type could have been present in Fig 1A The absence of dark spots within the gallbladder in Fig 1B indicates that these radiolucent gall stones were not present

Cholecystography was discovered by the late Dr Evarts Graham in 1924 (6–12) One evening in 1922, while contemplating the pharmacological observations of Abel and Rowntree (13) that phenol tetrachlorphthalein was eliminated almost entirely in the bile, Dr Graham conceived the idea of replacing the chlorine with other atoms with greater radiopacity to obtain an X-ray shadow of the gallbladder (12) It could be said that cholecystography was discovered by accident The sodium salt of tetraiodophenolphthalein was injected intravenously into six dogs A faint gallbladder picture became visible in only one of the six dogs It was discovered later that the five dogs which did not show a gallbladder picture had been fed on the morning of the experiment, whereas the one dog which did show a gallbladder shadow had been accidentally overlooked The severe toxic reactions encountered with tetraiodophenolphthalein caused Dr Graham to turn to tetrabromphenolphthalein which was found to be better tolerated Dr Graham's first published pictures of the gallbladder in the dog and in man were made with tetrabromphenolphthalein (6) The earlier toxic reactions with tetraiodophenolphthalein were found to be due to impurities which were eventually eliminated, thus making it possible to use the more radiopaque halogenated phenolphthalein

Tetraiodophenolphthalein was given in the form of the sodium salt (iodophthalein sodium, U S P Iodeikon[2]) as a bright blue solution by either intravenous injection or oral administration Administered orally, its taste was nauseating If the patient was able to keep it down, much of the dose was lost through diarrhea It was more frequently administered intravenously in order to circumvent the bitter taste and inaccuracy of dosage due to vomiting and diarrhea Even though it was administered by slow intravenous infusion, the incidence of nausea and vomiting still remained high In spite of the frequency and intensity of side effects, iodophthalein remained the cholecystographic contrast medium of choice for nearly twenty years

The first real improvement in cholecystography resulted from the studies of Dohrn and Diedrich (14), Kleiber (15), and Junkmann (16) in Germany in 1940 with the compound β (3,5 diiodo 4 hydroxyphenyl) α phenyl propionic acid, which they called Biliselectan[3] Shortly thereafter, this compound was introduced into this country with the name iodoalphionic acid, U S P, or Priodax[4] Although there was no great reduction in nausea and vomiting, it rapidly replaced iodophthalein in cholecystography largely because it could be administered simply in tablet form with greater convenience for both the radiologist and the patient (17–23)

Perhaps by tradition, the dog had become the chosen animal for the experimental investigation of compounds designed for cholecystography In 1946, when I first became interested in this field, Epstein, *et al* (24), had just published their observations on the potential use of the frog, chick, rabbit, and dog as test animals for cholecystographic media and concluded that the dog was the most satisfactory of this group However, they pointed out, and later emphasized (25), that compounds which gave the best results in the dog did not necessarily give the

[2] Product of Mallinckrodt Chemical Works St Louis Mo
[3] Product of Schering A G Berlin Germany
[4] Product of Schering Corporation Bloomfield N J

best gallbladder shadows in man. Jones, *et al.* (26), and later, Margolin, *et al.* (27), considered the dog the most satisfactory of the laboratory animals for the study of cholecystographic media. Our own investigations indicated that the mouse was too small, the rabbit and guinea pig gave inconsistent shadows, and the cat and dog gave reasonably consistent gallbladder shadows. Since there was no previous evidence to indicate that earlier investigators had tried the cat, it was decided to explore more fully the use of this species, in view of the reported discrepancies in the dog data (28). It was found that the cat was capable of discriminating among small changes in dosage and when the effect, in terms of density of the gallbladder shadow, was plotted against the logarithm of the dose, the relationship was linear in nature, thus satisfying the basic requirements of a biological assay (28–30). The cat offered an even more practical advantage over the dog in that its weight was about one-fifth that of the dog so that approximately five times as much information could be obtained from the same amount of test compound.

Method.—The details of the test procedure using the cat have been described previously (30). The interpretation of the cholecystogram and the assignment of an adjective rating in terms of a numerical equivalent, called the Cholecystographic Index (CI), is illustrated in Fig. 2. After each film is read, the CI values are averaged for a given dose level and designated the Average Cholecystographic Index (ACI) for that dose.

Experimental.—The use of this test method in the study of three different series of compounds is illustrated in Fig. 3. In series A, the compound with R = hydrogen, is iodopyracet, U. S. P., or Diodrast,[5] which is a well-known intravenous urographic contrast medium. This compound failed to produce any roentgenographic evidence of gallbladder visualization at 100 mg./Kg. intravenously in the cat. With R = methyl, very faint evidence of gallbladder concentration became apparent. When R = propyl, the gallbladder and urinary bladder appeared with almost equal intensity, indicating approximately an equal distribution between urinary and biliary excretion. Maximum gallbladder visualization was reached when R = butyl, or a total of six carbons in the alkanoic acid side chain. The density of the gallbladder shadows decreased gradually with R = amyl and hexyl, and dropped sharply at R = heptyl. The acute intravenous toxicity of this series of compounds in the mouse remained at about 6 Gm./Kg. with R = hydrogen and methyl, doubled at R = propyl, and continued to double with the addition of each methylene group, so that by the time fair to good gallbladder visualization was obtained, acute toxicity had increased to a point where it was likely to interfere with the clinical use of these compounds by this route of administration.

Turning to oral administration, it was found that a similar relationship existed between gallbladder visualization and chemical structure. More important, the intensity of the gallbladder shadow at a given dose level following oral administration appeared to be the same as that obtained by intravenous injection. Although the rate of appearance of the gallbladder shadow was slower in some instances by the oral route, this was offset by a marked reduction in systemic toxicity. The similarity between chemical structure and gallbladder visualization in the cat by both intravenous and oral administration is illustrated by series A and B in Fig. 3. Observations on more than 100 diiodo compounds indicated that a cholecystographic contrast medium consisted of two essential parts: (*a*) a firmly bound iodine-bearing nucleus to provide radiopacity and (*b*) a side chain containing a carboxyl group and an optimal size lipophilic group to provide for absorption and to direct excretion into the bile (28–34). Gallbladder visualization in the cat was obtained when the number of carbons in the side chain ranged from three to eight carbons in agreement with the findings of Epstein, *et al.* (24), in the dog. It was found that an iodinated phenyl nucleus could contain an amino group, a hydroxyl, or no substituent other than the iodines (32). The phenolic hydroxyl, which occurs in iodophthalein and iodoalphionic acid, was not found to be essential for gallbladder visualization as suggested by Epstein, *et al.* (24), and Natelson, *et al* (35). The nature of the side chain appears to be the most critical factor in determining whether gallbladder or urinary bladder visualization predominates. Where the alkanoic acid side chain contained less than three carbons, excretion occurred primarily by way of the kidneys. When the length of the side chain exceeded seven carbons, the compound tended to remain unabsorbed in the gastrointestinal tract. As each of several series of diiodo compounds was studied, maximum gallbladder visualization was found to be equal to but not much better than that observed with the diiodo reference compound, iodoalphionic acid, U. S. P.

Efforts to improve visualization by changes both in the radiopaque nucleus and in the side chain had not been successful. It occurred to me that perhaps the solution to this problem might be as simple as adding a third bulb to a light fixture. If the same number of molecules could be concentrated in the bile using a compound containing three iodine atoms instead of two, the triiodo compound should produce a brighter gallbladder picture. Among the various test compounds was one containing three iodine atoms. This compound, the ethyl-substituted triiodo structure shown in series C in Fig 3, had been synthesized by Mr. T. R. Lewis and Dr. Sydney Archer of the Organic Chemistry Division of the Sterling-Winthrop Research Institute (36). To test this hypothesis, I took a 3-Gm. dose in the evening with a fat-free meal. After X-ray the next morning, I was elated to find that, indeed, the density of my own gallbladder shadow was distinctly greater than that obtained in earlier studies with diiodo compounds. In the cat, this compound produced good to excellent visualization of the gallbladder in comparison with fair to good visualization with the best of the diiodo compounds. As both the lower and higher members of this series were prepared and tested, they were found to distribute themselves in the form of a pyramid with the original ethyl derivative as the most effective compound for visualizing the cat gallbladder (see series C, Fig. 3) In contrast to the findings among the diiodo compounds, peak visualization was observed in the 3-amino-2,4,6-triiodophenyl alkanoic acid series with

[5] Product of Winthrop Laboratories, New York, N. Y.

GRADING SCHEME

CHOLECYSTOGRAPHIC INDEX (CI)	ADJECTIVE RATING	DESCRIPTION
0	None	No visualization
1	Poor	Faint evidence of gallbladder concentration
2	Fair	Faint but visible outline of gallbladder
3	Good	Distinct outline with good density, and delineation of gallbladder
4	Excellent	Sharp outline of gallbladder with density demonstrating brilliant contrast with tissues

Fig. 2.—Cholecystographic screening procedure in the cat.

total of five carbons in the side chain. On the basis of these observations, the ethyl triiodo derivative was selected for further study as a potential cholecystographic contrast medium which later became known as iopanoic acid, U. S. P., or Telepaque.[1]

Cat *vs.* Dog.—Still unanswered was the question as to whether the cat or the dog provided an answer which could be relied upon in predicting which compounds might be useful in visualizing the gallbladder in man. The ACI for iopanoic acid was determined in both the cat and dog in comparison with iodophthalein, iodoalphionic acid, and iophenoxic acid[6] with results as summarized in Table II.

The structural formulas of these compounds and their respective dose response curves are shown in Fig. 4. Iodophthalein was clearly the least effective of the four compounds in visualizing the gallbladder in both species. Interestingly enough, it was distinctly more effective in visualizing the gallbladder of the dog than it was in the cat. For convenience in analyzing the data, a value indicating good visualization of the gallbladder, ACI_3, was derived from each of the curves except that for iodophthalein which was too ineffective. The ACI_3 values in the dog indicated the following order of activity, from most to least active: iophenoxic acid, iopanoic acid, and iodoalphionic acid. The ACI_3 values in the cat, on the other hand, indicated that iopanoic acid was the most effective, iodoalphionic acid the least effective, and the activity of iophenoxic acid was intermediate between these two. The early clinical studies on iopanoic acid indicated that the density of the human gallbladder shadow was distinctly greater and the incidence of side effects less than

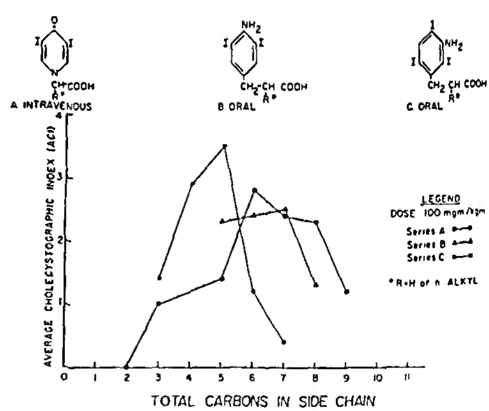


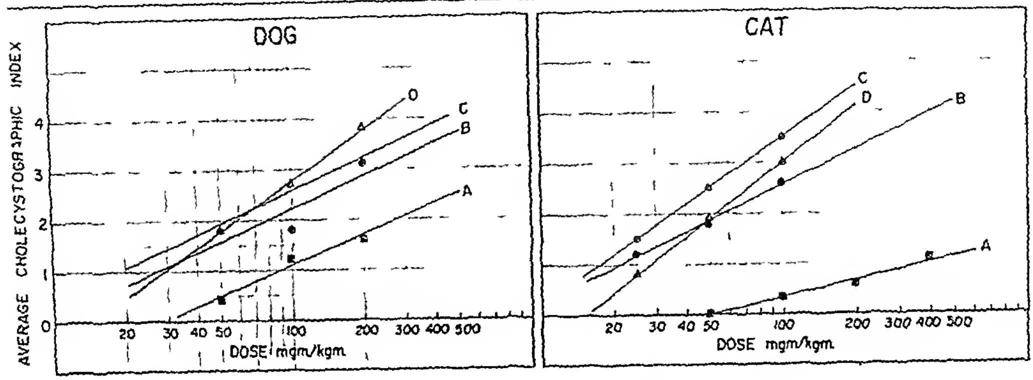
Fig. 3.—The relationship between chemical structure and gallbladder visualization following intravenous and oral administration of iodinated organic compounds in the cat.

that observed with iodoalphionic acid (37–54) Morgan and Stewart (40) observed that the density of the gallbladder shadow with iopanoic acid in man was approximately 1.35 times as great as that observed with iodoalphionic acid. This figure is in remarkably close agreement with the initial value, derived from the ACI's for these two compounds in the cat, which indicated that iopanoic acid was approximately 1.4 times as effective as iodoalphionic acid in visualizing the cat gallbladder (29). Iophenoxic acid, described by Dr. Domenic Papa, a previous recipient of this award, and his associates (55, 56) was found, in man, to produce gallbladder shadows which were less dense than those observed.

[1] Product of Schering Corporation, Bloomfield, N. J.

TABLE II.—ORAL CHOLECYSTOGRAPHY IN THE CAT AND DOG

Species,	Dose, mg./Kg	Iopanoic Acid No of Animals	Iopanoic Acid ACI	Iodophthalein No of Animals	Iodophthalein ACI	Iodoalphionic Acid No of Animals	Iodoalphionic Acid ACI	Iophenoxic Acid No of Animals	Iophenoxic Acid ACI
Cat	25	106	1.5	.	.	40	1 2	40	0 8
Cat	50	108	2 5	5	0	40	1 8	40	1 9
Cat	100	115	3 5	20	0 35	40	2 6	40	3 0
Cat	200	..	.	20	0.6	..			
Cat	400	.	.	15	1 1	..	..		.
Dog	50	6	1.8	5	0 4	5	1 8	5	1 8
Dog	100	7	2.7	5	1 2	8	1.8	6	2 7
Dog	200	7	3 1	5	1 6	8	3 1	6	3 8



COMPOUND	DOG ACI_3 mgm/kgm	DOG RELATIVE ACTIVITY	CAT ACI_3 mgm/kgm	CAT RELATIVE ACTIVITY
Iopanoic Acid	157	1 0	70	1 0
Iodoalphionic Acid	230	0 68	155	0 45
Iophenoxic Acid	117	1 34	100	0 70

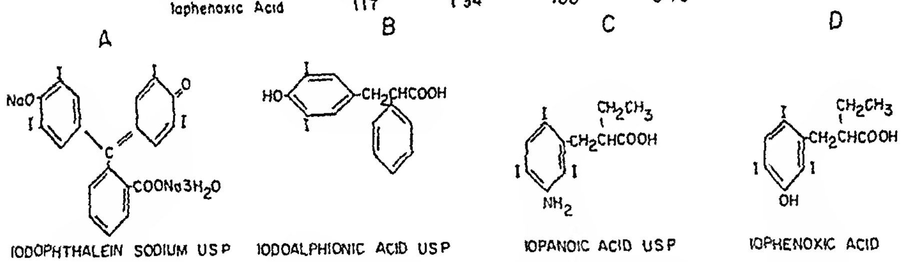


Fig. 4—A comparison of the oral cholecystographic activity of iodophthalein, iodoalphionic acid, iopanoic acid, and iophenoxic acid in the cat and dog.

with iopanoic acid, and intermediate in density between those of iopanoic acid and iodoalphionic acid (57–60). It can be concluded, therefore, that the cat proved to be a more reliable test animal than the dog for predicting the effectiveness of these compounds in visualizing the human gallbladder.

A frequent reminder of the early work with iodophthalein in cholecystography is the use of the word "dye" synonymously with the term X-ray contrast medium. It is true that iodophthalein possesses dye properties, but this fact has nothing to do with its ability to produce an image on X-ray film. None of the newer radiopaques are dyes. The use of the word dye in place of radiopaque or X-ray contrast medium to describe them is incorrect and should be discontinued.

UROGRAPHY

Urography is the roentgenographic examination of the urinary tract with the aid of an X-ray contrast medium. Retrograde urography involves the instillation of the radiopaque into the urinary tract by way of the urethra. Excretion urography involves the intravenous injection of a radiopaque which is rapidly excreted in the urine in sufficient concentration to cause the urinary tract to become visible on X-ray film. The present discussion is concerned only with excretion urography.

The early concepts leading to the use of iodinated organic compounds for excretion urography originated in Europe at approximately the same time as the discovery of cholecystography. In 1921, Professor Arthur Binz of Berlin, Germany, conceived the idea of synthesizing heterocyclic compounds containing iodine or arsenic to improve the therapeutic efficiency of the treatment of syphilis (61). Professor Binz and his assistant, Dr. Räth, synthesized some 700 new arsenic and iodine compounds, some of which were found to have a surprisingly low systemic toxicity. One of these, N-methyl-5-iodo-2-py-

done, while under investigation as a chemotherapeutic agent in the treatment of streptococcus infections, was found to be eliminated partly in the bile and, to a larger extent, in the urine (62) These observations led to the suggestion that it might be a useful X ray contrast medium (63) Swick found that this compound produced a distinct shadow of the urinary tract but failed to visualize the gallbladder of the rabbit (63) Better tolerance and greater water solubility was achieved with sodium 5 iodo 2 pyridone N acetate which Professor Binz selected for investigation in excretion urography (61) This compound, called Uroselectan,[7] was found to be an effective urographic contrast medium by Swick (63, 64), von Lichtenberg and Swick (65), and Jaches (66) Uroselectan was quickly replaced by two more effective diiodo pyridone derivatives, iodopyracet (Diodrast[5]) and sodium iodomethamate (Neo Iopax[8]) (67, 68) Sodium iodomethamate and iodopyracet, which was studied initially as the sodium salt and later as the diethanolamine salt (69-71), became the urographic contrast media of choice and remained unchallenged for nearly twenty years

Another most fascinating suggestion for excretion urography was the proposal by Dr Moses Swick in 1933 of the use of an organic nucleus representing a normal product of animal metabolism as a carrier for the radiopaque element necessary for the X ray visualization of the urinary tract (72, 73) The compound suggested was sodium *ortho* iodohippurate, a halogen derivative of a substance normally found in urine Contrary to initial expectations, this compound (iodohippurate sodium or Hippuran[9]) did not become a widely accepted urographic contrast medium in comparison with either sodium iodomethamate or iodopyracet Swick's idea of using a product of normal detoxication as a carrier for a radiopaque for intravenous urography lingered for many years in the imagination of Dr Vernon Wallingford, also a previous recipient of this award, and finally in 1952 emerged in the form of a new compound, sodium 3 acetamido 2,4,6 triiodobenzoate, sodium acetrizoate or Urokon[10] (74-76) The acute intravenous toxicity of sodium acetrizoate in the mouse was less than that of either iodopyracet or sodium iodomethamate

For many years we had been testing numerous iodinated organic compounds for acute intravenous toxicity in the mouse in an effort to find one with an acute intravenous LD_{50} value greater than the 4 to 6 Gm /Kg level of sodium iodomethamate and iodopyracet, respectively In 1952, this level had been raised to 8 Gm /Kg with the discovery of sodium acetrizoate In 1954, Dr Aubrey Larsen of the Organic Chemistry Section at the Sterling Winthrop Research Institute, succeeded in shattering this toxicity barrier when he described the synthesis of sodium 3 5 diacetamido 2,4,6 triiodobenzoate, diatrizoate sodium or Hypaque[11] (77-79) The acute intravenous toxicity of diatrizoate sodium in the mouse was found to be approximately 14 Gm /Kg, which indicated a substantial elevation of the systemic toxicity ceiling for compounds in the field of excretion urography On the basis of the acute toxicity observations in the mouse, this compound was selected for further laboratory evaluation as a potential urographic contrast medium Independently and unknown to us, this compound was described at about the same time in Germany by Langecker, *et al* (80), and studied clinically in the form of a mixture of the sodium and N methylglucamine salts in Germany as Urografin[12] (81, 82), and later in this country as Renografin[13] (83, 84)

Method —The test method evolved for searching for an improved contrast medium for urography consisted of two parts (*a*) acute intravenous toxicity determination in the mouse and (*b*) excretion urography in the rabbit

(*a*) *Acute Intravenous Toxicity* — As pointed out above, an arbitrary criterion of 4 to 6 Gm /Kg had been established for the selection of compounds for evaluation in respect to experimental urography in the rabbit Those compounds found to be effective urographic media in the rabbit in preliminary tests were then subjected to acute intravenous toxicity determinations in animal species other than the mouse, while continuing the urographic studies in the rabbit The species used for these acute toxicity determinations included the rat, rabbit, cat, and dog Since the acute lethal dose cannot be determined in man, it must be assumed that the systemic toxicity for man could be equivalent to the lowest LD_{50} value found among the various animal species The compound demonstrating the highest LD_{50} value and the least variation among animal species is most likely to be the safest compound to use in man The method for determining acute intravenous toxicity in the various species, numbers of animals used, concentrations, and rates of injection employed in the study of diatrizoate sodium in comparison with iodopyracet, sodium iodomethamate, and sodium acetrizoate have been described previously (78)

(*b*) *Urography* —Adult, healthy, New Zealand white rabbits weighing 2 5 to 3 5 Kg are maintained in individual cages in a well lighted, air conditioned animal room on a nutritionally adequate diet with a maximum allowance of 500 cc of water per day Because of the importance of accurate timing of the serial X-ray exposures, each rabbit is studied individually For a given test, a group of five rabbits is fasted for forty eight hours to eliminate food shadows over the kidney area and denied access to water for twenty four hours prior to the injection of the test compound On the morning of the test, a control X ray picture is taken of each rabbit to determine the radiographic status of the urinary tract area Only those rabbits showing an abdominal field relatively free of gas and food shadows are used The rabbit selected for testing is fastened supine, in position under the X ray tube A cloth compression band is applied firmly just above the symphysis pubis immediately before injection with the first cassette in position under the rabbit The test compound, in neutral aqueous solution, is injected intravenously at a rate of 12 cc /minute at a dose of 100, 200 400, 800, 1,600, or 3,200 mg /Kg

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[7] Product of Schering A G Berlin Germany
[8] Product of Schering Corporation Bloomfield N J
[9] Product of Mallinckrodt Chemical Works St Louis Mo
[10] Product of Mallinckrodt Chemical Works St Louis Mo

[11] Product of Winthrop Laboratories New York N Y
[12] Product of Schering A G Berlin Germany
[13] Product of E R Squibb & Sons New York N Y

X-ray pictures are then taken at five and ten minutes after injection. The compression band is immediately released after the ten-minute picture and reapplied at ten minutes before the next X-ray pictures at twenty, forty, and eighty minutes after injection At the higher dose levels, X-ray pictures are taken without compression at hourly intervals up to seven and twenty-four hours, and daily thereafter until evidence of complete elimination has been achieved. A minimum of five animals is used for each dose level. The same procedure is used for urographic examination in the cat and dog with the exception that fasting is limited to twenty-four instead of forty-eight hours.

The urograms are read and interpreted in accordance with the grading scheme shown in Fig. 5. The interpretation of the film is based on the ability of the compound to delineate the morphology of the upper urinary tract, the highest score being assigned when the calices, pelves, and ureters are visible as indicated in part A of Fig. 5. The quality of the shadow is also taken into consideration and assigned a numerical value as indicated in part B of Fig. 5. The X-ray pictures across the top of Fig. 5, from left to right, show the control plate before injection; visualization of the calices, pelves, and a faint outline of the upper ureters at 5 minutes; maximum visualization of these structures at ten minutes; very little radiopaque urine visible in the pelves and ureters at twenty minutes, and only a trace visible in the pelves at forty minutes, with the major portion of the injected dose visible in the urinary bladder. The urographic score is determined for each film and designated as the Urographic Index (UI). As additional animals are tested at each dose level, the urographic indexes for each time interval are averaged and designated as the Average Urographic Index (AUI). The animals are returned to the regular colony after completion of the urographic series of films and are not used again for a period of at least one week

Experimental.—The use of this test procedure in the evaluation of the urographic properties of diatriazoate sodium in comparison with those of iodopyracet in the rabbit is shown in Table III.

Urography.—Maximum visualization of the urinary tract of the rabbit was observed with diatrizoate sodium at each dose level from 100 to 1,600 mg./Kg., at ten minutes after injection. Maximum visualization was observed with iodopyracet at ten minutes with 100 and 200 mg./Kg., at twenty minutes with 400 and 800 mg./Kg., and at ten minutes with 1,600 mg./Kg., indicating a somewhat slower rate of visualization of the urinary tract with iodopyracet The AUI for diatrizoate sodium was greater than that observed for iodopyracet at each dose level except the 200 mg./Kg. dosage, indicating that diatrizoate sodium produced a better diagnostic picture of the urinary tract of the rabbit than iodopyracet at equivalent dose levels. It was found that dosages of 400 to 800 mg./Kg. of diatrizoate sodium and 800 mg./Kg. of iodopyracet were required in order to achieve good diagnostic visualization of the upper urinary tract in the rabbit. The AUI for diatrizoate sodium continued to increase in magnitude with increase in dosage up to 7,960 mg./Kg. where mortality began to appear. The AUI for iodopyracet, on the other hand, increased to a value of 45.0 at 800 mg./Kg., after which it showed no further important increase with increase in dosage. A 20% mortality was encountered with diatrizoate sodium at 7,960 mg./Kg. A 40% mortality was encountered with

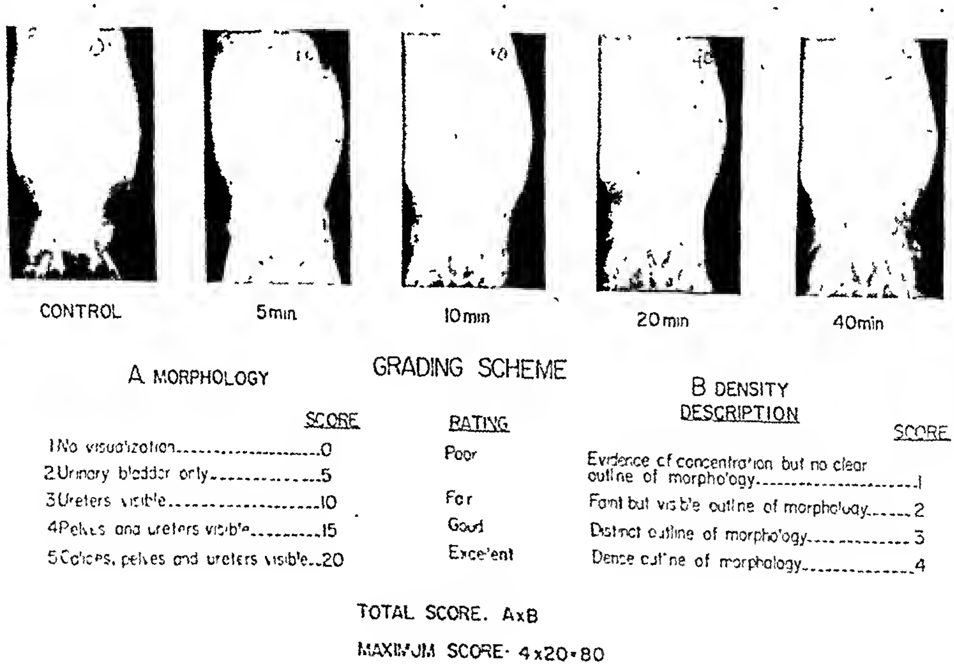

Fig. 5.—Urographic screening procedure in the rabbit

TABLE III —INTRAVENOUS UROGRAPHIC PROPERTIES OF DIATRIZOATE SODIUM AND IODOPYRACET IN THE RABBIT

Compound	Dose, mg / Kg	No of Rabbits	——Average Urographic Index (AUI) at Intervals After Injection, Minutes—— 5	10	20	40	80	120	180	240	300	360	420	Mortality
Diatrizoate sodium	100	14	21 8	29 0	21 0	15 3	9 3							0/14
Diatrizoate sodium	200	19	26 4	27 8	26 6	18 7	10 8				.	.	..	0/19
Diatrizoate sodium	400	17	32 6	37 0	31 2	20 6	18 2				..	.	..	0/17
Diatrizoate sodium	800	17	49 7	61 2	44 6	40 0	24 0					.		/017
Diatrizoate sodium	1,600	16	37 0	57 5	55 0	38 8	26 2						.	0/16
Diatrizoate sodium	3,200	21	40 5	62 6	69 6	64 0	62 5	66 8	46 0	33 3			13 3	0/21
Diatrizoate sodium	6,400	5	28 0	48 0	52 0	64 0	64 0	76 0	76 0	60 0	40 0	47 5	26 0	0/5
Diatrizoate sodium	7,960	5	36 0	44 0	50 0	50 0	57 5	70 0	80 0	70 0	50 0	25 0	25 0	1/5
Diatrizoate sodium	10,000	7	51 5	56 0	60 0	64 0	72 0	75 0	75 0		37 5	37 5	30 0	3/7
Iodopyracet	100	5	14 0	19 0	18 0	9 0	10 0							0/5
Iodopyracet	200	9	20 0	31 2	19 4	11 7	10 0						..	0/9
Iodopyracet	400	16	18 1	19 0	22 2	19 4	18 7						.	0/16
Iodopyracet	800	6	28 4	40 0	45 0	40 0	30 0							0/6
Iodopyracet	1,600	5	26 0	34 0	34 0	34 0	24 0					.	.	0/5
Iodopyracet	3,200	5	20 0	24 0	33 0	39 0	52 0							0/5
Iodopyracet	3,980	5	20 0	20 0	28 0	28 0	40 0	46 7		40 0	23 3	23 3	30 0	2/5
Iodopyracet	5,000	5	28 0	28 0	28 0	26 6	40 0	60 0		60 0	60 0	60 0		4/5
Iodopyracet	6,400	5	20 0	24 0	26 0	25 0								5/5

iodopyracet at 3,980 mg /Kg , indicating that diatrizoate sodium was approximately half as toxic as iodopyracet under the conditions of excretion urography in the rabbit

An analysis of the urographic properties of diatrizoate sodium in comparison with those of iodopyracet at 800 mg /Kg intravenously in the rabbit is illustrated in Fig. 6 The quality of morphologic delineation of the upper urinary tract increased rapidly to a maximum at ten minutes after injection of diatrizoate sodium Visualization with iodopyracet increased more slowly to a maximum at twenty minutes after injection The greater magnitude of the maximum urographic score with diatrizoate sodium indicates a more effective visualization of

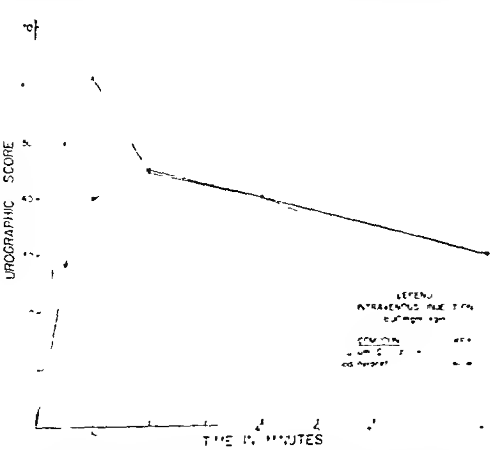

Fig. 6.—A comparison of the urographic properties of diatrizoate sodium and iodopyracet in the rabbit.

the upper urinary tract of the rabbit than was obtained with iodopyracet.

Acute Toxicity.—The acute intravenous LD_{50} values for diatrizoate sodium in comparison with those for iodopyracet, sodium iodomethamate, and sodium acetrizoate in five animal species are summarized in Fig. 7 New data included in Fig. 7 are acute intravenous LD_{50} data for iodopyracet and sodium iodomethamate in the rabbit, cat, and dog The methods and number of animals used are identical to those described earlier (78). The acute intravenous LD_{50} for iodpyracet was found to vary from 3 Gm /Kg to about 6 Gm /Kg , and for sodium iodomethamate from 4 Gm /Kg to about 7 Gm /Kg Although the acute intravenous toxicity of sodium acetrizoate was approximately 8 Gm /Kg. in the mouse, the values for the rat, rabbit, cat, and dog ranged between 5 and 6 Gm /Kg The acute intravenous LD_{50} for diatrizoate sodium was found to range from 11 3 Gm /Kg in the cat to 14 0 Gm /Kg in the mouse, indicating an order of systemic toxicity in the various animal species of about half that of iodopyracet, sodium iodomethamate, or sodium acetrizoate.

The laboratory evidence of improved urographic visualization and, in particular, the marked reduction in systemic toxicity of sodium 3,5-diacetamido-2,4,6-triiodobenzoate suggested that this compound merited investigation as a urographic contrast medium in man The first clinical studies with diatrizoate sodium, carried out by the late Dr. T. D Moore, who had also conducted the first clinical investigations with iodopyracet in this country (70), demonstrated a distinct improvement in the diagnostic quality of the urograms along with a low rate of mild side reactions in general agreement with the experimental data in laboratory animals (85) Subsequent clinical studies indicated improved diag-

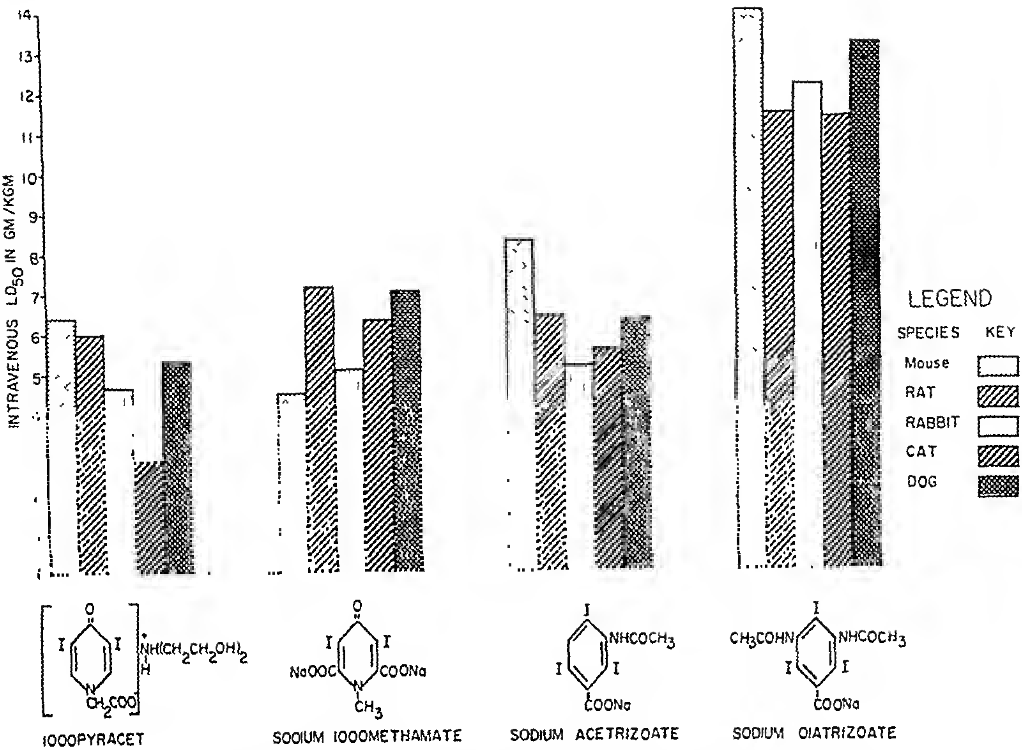

Fig. 7.—Acute intravenous toxicity data on iodopyracet, sodium iodomethamate, sodium acetrizoate, and sodium diatrizoate in the mouse, rat, rabbit, cat, and dog.

nostic visualization and a reduced incidence of side effects in comparison with iodopyracet, sodium iodomethamate, or sodium acetrizoate (86–102).

One of the most interesting observations to emerge from the laboratory studies on diatrizoate sodium was the finding that iodine could be incorporated into the sodium 3,5-diacetamidobenzoate molecule with no increase in toxicity when measured in terms of mM/Kg. by intravenous injection in the mouse (78). This was also found to be true in the case of sodium acetrizoate, as shown in Fig. 8 These findings suggested a possible explanation for the limited success of Dr. Swick's proposed use of iodohippurate sodium in urography (72, 73). As seen in Fig. 8, the acute intravenous toxicity of sodium benzoate in the mouse was reduced to one-half by conjugation with glycine. In contrast to the observations on the effects of iodine substitution in sodium 3,5-diacetamidobenzoate or sodium 3-acetamidobenzoate, the incorporation of iodine into sodium hippurate resulted in a twofold increase in toxicity when measured in terms of mM/Kg. of body weight in the mouse.

SUMMARY

1. The properties of radiopaques were discussed in general and those of iodinated organic compounds used as radiopaques in cholecystography and urography in particular.

2. The development of test procedures for the evaluation of radiopaques for cholecystography and urography were described.

3. Relative oral cholecystographic activity, in order of decreasing effectiveness, was observed in the dog: iophenoxic acid, iopanoic acid, iodoalphionic acid, and iodophthalein, and in the cat: iopanoic acid, iophenoxic acid, iodoalphionic acid, and iodophthalein. The relative order of activity observed in the cat resembled that described in the literature for man, indicating that the cat was a more satisfactory test animal than the dog for the estimation of the cholecystographic activity of these compounds.

4. The essential components of a cholecystographic medium appear to consist of (*a*) an iodinated aromatic nucleus to provide radiopacity and (*b*) a "transport mechanism" consisting of an alkanoic acid side chain containing 6 to 7 carbons with 2 iodines in the aromatic nucleus and 5 carbons with 3 iodines in the radiopaque portion of the molecule.

5. The urographic properties of diatrizoate sodium were compared with those of iodopyracet in the rabbit. Diatrizoate sodium was found to produce earlier and better visualization of the upper urinary tract with less than half the systemic toxicity observed with iodopyracet.

6. The acute intravenous toxicity of diatrizoate sodium in the mouse, rat, rabbit, cat, and dog was found to be approximately half as grea

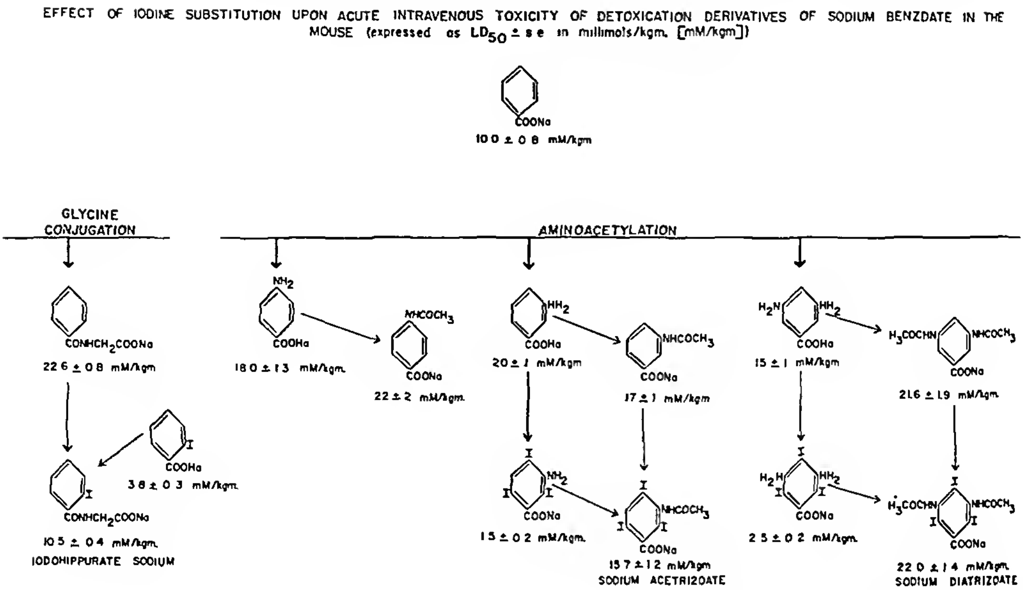

Fig. 8.—The acute intravenous toxicity of iodohippurate sodium, sodium acetrizoate, and sodium diatrizoate, expressed in terms of mM/Kg. in the mouse, before and after the incorporation of iodine (78) (Reproduced by permission of The Williams & Wilkins Co)

as that observed for iodopyracet, sodium iodomethamate, or sodium acetrizoate.

7. The incorporation of iodine into both sodium 3-acetamidobenzoate and sodium 3,5-diacetamidobenzoate had no effect on toxicity when measured in terms of mM/Kg. by intravenous injection in the mouse, indicating that radiopacity had been conferred upon these molecules with no expense in terms of systemic toxicity.

8. The urographic contrast medium also appears to consist of (*a*) an iodinated aromatic nucleus to provide radiopacity and (*b*) a carboxyl group or, at most, an alkanoic acid side chain with no more than three carbons to provide water solubility.

9. The use of both test procedures in the search among iodinated organic compounds for improved X-ray contrast media was illustrated by a description of the emergence of iopanoic acid and diatrizoate sodium from unknown chemical compounds in the laboratory to useful diagnostic aids to the radiologist in the form of individual medicinal entities known as Telepaque and Hypaque, respectively.

CONCLUSION

I would like to point out that in the search for a new medicinal agent, a research idea is much like a new-born infant; it demands a great deal of expert help from many sources. The process of creating a new medicine is complicated and requires the skills of many highly trained people functioning as a closely coordinated team. I have been extremely fortunate in having the opportunity to work with a most stimulating group of research investigators at the Sterling-Winthrop Research Institute. I am deeply grateful to Dr. M. L. Tainter, our Director of Research, whose encouragement and guidance has been a great source of help and inspiration to me. I am particularly grateful to Dr. Sydney Archer and Dr. Aubrey Larsen, whose chemical ingenuity has made these studies possible. I wish to thank the many members of the Chemistry and Biology Divisions who have helped in the successful completion of these studies. I would like to emphasize especially the importance of the contributions of the research staff of our Pharmacy Division, whose specialized technical training has helped solve the many problems involving compounding, solubility, stability, and formulation. I would also like to acknowledge, with grateful appreciation, the technical assistance of Mr. John Romano, Mr. Armand Brousseau, Mr. Leon Duprey, and Miss Nancy Harvey. Finally, I wish to express my sincere thanks to the Chilean Iodine Educational Bureau, Inc., for the honor of having been named recipient of their 1957 Iodine Research Award.

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A New Highly Reactive Aluminum Hydroxide Complex for Gastric Hyperacidity I*

In Vitro Rate of Acid Neutralization

By E. T. HINKEL, Jr., M. P. FISHER, and M. L. TAINTER

A new, highly reactive form of aluminum hydroxide polymer has been developed which takes up acid faster and in larger amounts than conventional aluminum hydroxide dried gel under conditions similar to those existing in the hyperacid stomach. Using this new form of aluminum hydroxide a tablet has been compounded which, in the recommended dose, neutralizes acid several times faster than comparable tablets in their therapeutic doses, at pH levels which will practically inactivate gastric pepsin. It also neutralizes about three times as much total acid as previously available tablets under these same conditions. The new tablet, as a result of this greater activity, should provide a more effective means of controlling gastric hyperacidity than previously available tablets.

Liquid aluminum hydroxide preparations have long been recognized as being highly effective in the management of gastric hyperacidity. However, from the standpoint of convenience to the patient it is much more desirable to use a dry dosage form, such as a tablet. Unfortunately, the process of drying these gels for such use may destroy much of the original beneficial effect. Investigation in these laboratories of the factors controlling this loss of activity has resulted in the development of a new, especially reactive polymeric aluminum hydroxide complex. The purpose of this paper is to present the antacid properties of a tablet prepared from this material in comparison with existing products.

Prior to the introduction of aluminum hydroxide products, the *in vitro* evaluation of gastric antacids consisted essentially of determining their total acid consuming capacity. The major ingredients of these antacids, such as sodium bicarbonate, calcium carbonate, magnesium carbonate, and the like, reacted rapidly and completely with either dilute acid or gastric juice. However, dry aluminum hydroxide preparations differ widely in their antacid power in both rate and completeness of reaction, and are greatly influenced by the reaction conditions. As a result, the total acid neutralizing power of such preparations may be practically meaningless as an indication of therapeutic efficacy.

Many *in vitro* methods for the evaluation of antacid products have been utilized (1–5). With few exceptions these procedures are modifications of those given by Johnson and Duncan (6) and Rossett and Flexner (7), in which acid is added to a given amount of antacid. The pH *versus* time is recorded. The aim of many investigators has been the development of a testing method which uses the equivalent of an artificial stomach. Some use artificial gastric juice or devices to withdraw portions of the "stomach" contents, and the like. In theory these are appealing. However, the variables encountered clinically, even in the same patient, are so numerous (8) that they make improbable an *in vitro* duplication of the human stomach.

As far as can be determined, the method developed by Rossett, *et al.*, has given the best *in vitro-in vivo* correlation (7, 9, 10, 11). Their main contribution has been recognition of the fact that dilute acid or gastric juice has to be added to the antacid continuously at a sufficiently rapid rate to simulate maximal secretion rates. Even the more refractory and therapeutically unsatisfactory aluminum hydroxide products will show respectable "activities" if acid addition is slow enough. Their method, then, is useful in measuring speed of reaction and indicating ability to hold gastric pH within desired limits.

A different approach to the problem has been taken by Bachrach (12) and by Gwilt (13). Their method consists essentially of adding dilute acid to the antacid at such a rate as to maintain a a pH of 3.5. This method gives a direct estimate of the speed of action and total available activity, whereas the previous procedures demonstrate primarily the buffering effect as measured by pH and duration of action.

Attention in this paper is directed to the fallacy of considering speed as the sole criterion of effectiveness. Were this the case, sodium bicarbonate and the alkaline-earth carbonates would not have fallen into disfavor. It is true that they react almost instantly with acid, but they buffer at too alkaline a pH where inhibition of peptic activity is complete and where acid rebound

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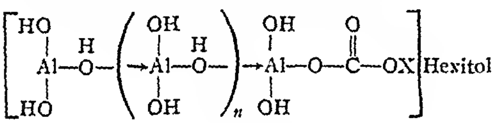
is likely. Therefore, it becomes necessary to establish, in addition to rate, the buffering range. A subsequent paper will deal with this aspect of the problem. However, the Bachrach method, as mentioned, is ideal for study of the rate and total activity and has proved very valuable in the development of a new, highly reactive aluminum hydroxide antacid tablet which is described in this paper.

EXPERIMENTAL

The materials studied were ten leading aluminum hydroxide antacid tablets. These contain aluminum hydroxide as their chief antacid ingredient, which in some is reinforced by addition of magnesium hydroxide, oxide, peroxide, or trisilicate, or calcium carbonate. They are identified in Table I as tablets 1–10. The new tablet, Creamalin tablets (Winthrop Laboratories), presented herein contains a new, highly reactive polymeric aluminum hydroxide hexitol complex in an amount equivalent to 320 mg. of aluminum hydroxide dried gel and 75 mg. of magnesium hydroxide. The structure of this polymer has not been completely elucidated. However, it can be represented by the partial formula



where n is at least 1 and averages less than 6, and X is a cation.

The procedure of Bachrach (12) was employed. Since many of the antacid tablets are intended to be chewed and do not disintegrate readily in aqueous media, all the tablets tested were ground in a mortar to pass a 60-mesh screen. Aliquots of the powdered antacid equalling the weight of an average tablet were suspended in 40 ml. of distilled water in a constant temperature (37°) cell equipped with a mechanical stirrer and pH electrodes. Ten drops of a 10% aqueous solution of a nonionic surfactant (Triton X-100) were added to insure wetting, since it was found that otherwise many products would not readily disperse. One-tenth normal hydrochloric acid was added continuously from a buret in just sufficient volumes to maintain the pH at 3.5. The volumes of acid added were recorded at frequent intervals for one hour. The end point, pH 3.5, is within the therapeutically desirable range, since at this level peptic activity is practically stopped, and the excessive acidity of the ulcer patient is reduced to nonirritating concentrations.

Since the products other than the Creamalin tablets were of indeterminate age when purchased, their stability was studied. There was no significant change in their activities after six months at room temperature, and, therefore, it was concluded that the results obtained initially represented a reliable measure of their usual activity.

Selection of Titrant. Although some investigators prefer to use an artificial gastric juice for their titrations, this study was carried out with 0.1 N HCl for two reasons. First, the presence of a protein can hinder the efficient operation of the glass electrode due to adsorption on the surface of the sensitive glass membrane (13). Second, the concentrations of enzyme, salts, etc., *in vivo* vary over too broad a range to permit the preparation of a truly representative artificial gastric juice.

Selection of Dosage.—There are a number of bases on which the various antacid tablets might be compared. These include tablet *vs.* tablet, by average label dose, by weight of active ingredients, or by equivalent total acid combining capacity. A tablet *vs.* tablet comparison has little meaning since the size

TABLE I.—PERTINENT DATA FOR THE ANTACID PRODUCTS EVALUATED

Product	Antacid Active Ingredients		Av. Tablet Wt., mg.	Label Dose, Tabs.	Av. Label Dose, Tabs.	Total Acid Consuming Capacity, ml. 0.1 N HCl per Tablet	Total Acid Consuming Capacity, ml. 0.1 N HCl per Gm. Active Ingredients	Number of Tablets to Neutralize 250 ml. 0.1 N HCl	Number of Tablets to Contain 1 Gm. Active Ingredients
New Creamalin	Aluminum hydroxide[a]	320 mg.	675	2–4	3.00	125	318	2.00	2.54
	Magnesium hydroxide	75 mg.							
No. 1	Aluminum hydroxide[a]	400 mg.	913	1–2	1.50	112	224	2.24	2.00
	Magnesium hydroxide	100 mg.							
No. 2	Aluminum hydroxide[a]	784 mg.	1055	0.5–1	0.75	225	288	1.11	1.28
No. 3	Aluminum hydroxide[a]	150 mg.	552	1–2	1.50	79	198	3.17	2.50
	Magnesium trisilicate	250 mg.							
No. 4	Aluminum hydroxide[a]	250 mg.	1262	1–4	2.50	87	115	2.88	1.32
	Magnesium trisilicate	500 mg.							
No. 5	Aluminum hydroxide[a]	400 mg.	795	1–2	1.50	170	283	1.47	1.66
	Magnesium oxide	200 mg.							
No. 6	Aluminum hydroxide[a] } Magnesium hydroxide }	400 mg.	467	2–4	3.00	84	210	2.97	2.50
No. 7	Aluminum hydroxide[a]	648 mg.	1355	0.5–2	1.25	189	195	1.32	1.03
	Magnesium trisilicate	324 mg.							
No. 8	Aluminum hydroxide[a]	144 mg.	602	1–2	1.50	101	336	2.48	3.33
	Calcium carbonate	71 mg.							
	Magnesium peroxide	86 mg.							
No. 9	Aluminum hydroxide[a]	97 mg.	398	3–5	4.00	60	151	4.16	2.52
	Magnesium trisilicate	292 mg.							
No. 10	Aluminum hydroxide[a]	300 mg.	800	2–4	3.00	80	266	3.13	3.33

[a] Calculated as aluminum hydroxide dried gel, U. S. P.

of the tablet is arbitrarily selected by the manufacturer. The comparison is more meaningful when based on the average dose recommended for the relief of gastric hyperacidity. Therefore, the first comparison was made in the present study on a weight of ground tablet equivalent to the average label dose.

In addition, comparison of equal weights of the antacid ingredients provides a measure of the inherent efficiency of the antacids selected, independent of tablet size or dosage recommendations. Therefore, a weight of tablet material representing 1.0 Gm. of the combined antacid ingredients of each tablet was calculated, and this information was used to prepare a second series of titration curves.

The third useful basis for comparison is derived from the total acid neutralizing power of the tablet mix. This can be measured readily by a determination of the total acid consuming capacity using the extreme conditions of the U. S. P. procedure (14). From these values the weights of tablet material can be selected which will have the same total acid combining power, e. g., 250 ml. of 0.1N HCl, and the comparisons of speed, etc., made at these equivalent weights.

In practice it is more convenient to use aliquots of the powdered antacid preparation to avoid adding excessive volumes of acid in the titrations. However, it was determined experimentally that for any given powder there is a direct relationship between the weight of tablet material titrated and the volume of acid required. Thus a simple calculation is all that is necessary to convert the values obtained for any given weight of material to those applicable to another quantity.

RESULTS

In Table I are summarized pertinent measurements on the eleven tablets included in this study. They comprise ten of the leading aluminum hydroxide antacid tablets and the new Creamalin tablet.

The weight of the combined antacid ingredients in the tablets varied from 300 to 960 mg. The total size of the tablets also showed a wide range because of the presence of diluents, binders, and nonantacid ingredients. The smallest was 398 mg. and the largest, 1,355 mg. The total acid neutralizing capacity of the tablets, as determined by the U. S. P. method, also varied widely. Tablet No. 9 neutralized only 60 ml. of acid in this test while the greatest capacity was shown by tablet No. 2, which neutralized 225 ml. of acid.

There was also calculated the number of tablets required to neutralize 250 ml. of acid under these conditions. The table shows that there was more than a three-fold difference in this measure of activity. The size of the tablet obviously modifies the amount of acid it can neutralize. However, it can be seen from the table that when the quantities of tablets required to contain 1.0 Gm. of active ingredients are compared, there is still more than a three-fold difference between the extremes.

The data illustrate quite clearly that the innate reactivity of aluminum hydroxide and related antacids varies widely among different preparations and is influenced strongly by factors other than tablet size and composition. In this measurement of total antacid power, which does not disclose speed or buffering power, the new Creamalin tablet occupied an intermediate position when considered on a tablet basis, because the tablet is smaller than about half those considered. When the data are considered in terms of capacity per Gm. of active ingredients, the new Creamalin tablet will be seen to have considerably more antacid power than any of the other tablets of similar composition.

The most important test, however, of potential clinical value is not the assay of total activity as described by the U. S. P., but rather the speed with which acid can be taken up while maintaining a pH which will inhibit peptic activity. The results of titrations of these materials, carried out to a constant pH as described above, are shown in Figs. 1, 2, and 3.

In Fig. 1 the activities per Gm. of antacid ingredients are plotted. It will be noted that the new Creamalin tablet neutralized acid faster than nine of the other tablets. It also neutralized more acid in the sixty-minute titration period than the others by a very appreciable margin. This test more nearly approaches physiological conditions than the U. S. P.

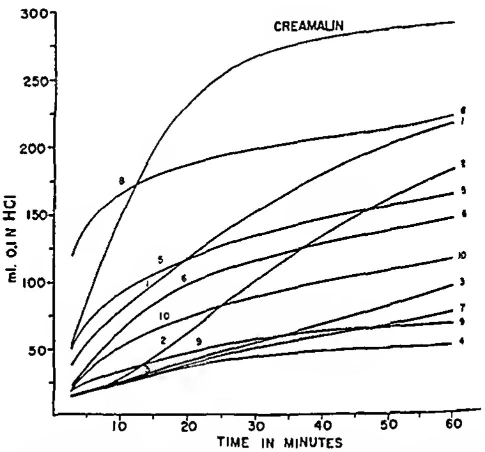


Fig. 1.—Rate of acid neutralization at pH 3.5 and 37°. Sample equals weight of tablet material containing 1.0 Gm. active ingredients.

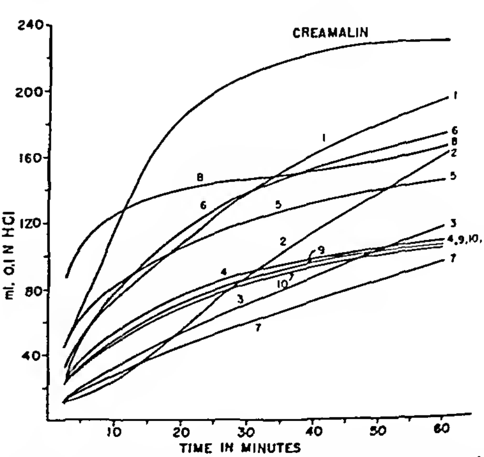


Fig. 2.—Rate of acid neutralization at pH 3.5 and 37°. Sample equals weight of tablet material that will neutralize 250 ml. 0.1 N HCl by U. S. P. method.

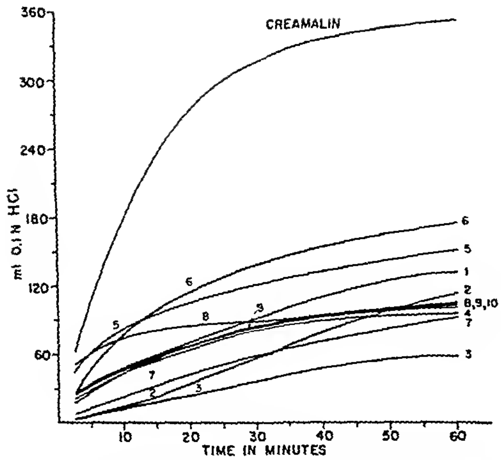


Fig. 3.—Rate of acid neutralization at pH 3.5 and 37°. Sample equals weight of tablet material representing average label dose.

procedure and hence has considerable significance in suggesting superior therapeutic activity for the new tablet.

It might be thought that this greater reactivity is the result of comparing the tablets on a weight rather than a chemically equivalent basis. Hence, in Fig. 2 are shown the values when the comparisons are made for quantities of the antacid which will neutralize 250 ml. of 0.1 *N* HCl under U. S. P. conditions. Again the superiority of the new Creamalin is manifest. By the end of thirty minutes, for example, the new Creamalin has exerted 85% of its theoretical antacid capacity. For the same time interval the next best was only up to 60%. For the forty-, fifty-, and sixty-minute intervals the ratios of Creamalin acid consumption (in per cent) to the next best were 90:65, 92:72, and 93:79, respectively.

In addition, the patient is interested in the extent and rapidity of his relief when taking the recommended dose. In Fig. 3 the values obtained using this very practical criterion are set forth. It will be noted immediately that when this usual clinical dosage is titrated, the new Creamalin was markedly superior. It reacted much more rapidly throughout the entire titration and ended up by neutralizing approximately three times as much as the other tablets. This suggests a decided therapeutic advantage at the recommended dosage in both onset of relief and in the total amount of acid neutralized, resulting in better control of gastric hyperacidity.

DISCUSSION

It is widely recognized that aluminum hydroxide dried gels from different sources vary considerably in their physical and chemical properties. The most important difference when considering them as antacids is, of course, reactivity with acid. It becomes extremely important, then, to define precisely what is meant by this reactivity. Albeit the mechanism of reaction between aluminum hydroxide and hydrochloric acid is rather poorly defined, it is quite easy to demonstrate experimentally certain variations depending on the conditions chosen.

When studying total acid consuming power, the classical assay methods utilize a large excess of hydrochloric acid to react with the antacid and back titrate to determine the amount consumed. As can be expected, the reaction is forcefully driven towards completion. In the light of the present therapeutic considerations the data thus obtained can be misleading. This is especially true in the case of those products whose rates of reaction change with time, for not only must a product neutralize the acid, but it must do so rapidly to achieve prompt relief. In other words, the rapidity of reaction is directly proportional to the slope of the titration curve. Of course, it is possible to effect a high initial velocity by including such ingredients as magnesium hydroxide, alkali bicarbonates, and alkaline-earth carbonates. Magnesium hydroxide has become quite popular for this purpose due to its attendant laxative properties. However, once these instantly reacting compounds are neutralized, the antacid effect becomes a function solely of the aluminum hydroxide present. The reactivity of the aluminum hydroxide, then, is the determining factor in the persistent efficacy of this type of antacid.

As mentioned, these differences are not readily apparent under the customary procedures of assay. Also the conditions prescribed do not represent the environment encountered by aluminum hydroxide in the stomach. Hence, when more physiological test conditions are set up, many of the antacid preparations are found to be much less effective than would be anticipated.

Guided by this new concept of therapeutic reactivity it has been possible to make a highly reactive complex aluminum hydroxide polymer which takes up acid quickly and in almost theoretical amounts under physiological conditions. From this has been formulated a new tablet which makes these new properties available therapeutically.

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A New Highly Reactive Aluminum Hydroxide Complex for Gastric Hyperacidity II*

In Vitro Buffering Range and Duration of Action

By E. T. HINKEL, Jr., M. P. FISHER, and M. L. TAINTER

A new, highly reactive polymeric aluminum hydroxide hexitol complex has been developed and incorporated into an antacid tablet. When tested in comparison with a liquid aluminum hydroxide gel of proved clinical effectiveness, the new Creamalin tablet has an almost identical duration of action and buffering range. When tested in comparison with ten leading aluminum hydroxide tablets using an *in vitro* method recognized as having good correlation with *in vivo* experience, the new Creamalin tablet exerts its buffering activity in the optimal range for much longer periods than the others. Effective antacid therapy has been defined as the elevation of gastric pH to between 3.0 and 5.0 with the optimum being 3.5 to 4.5. The new Creamalin tablet should provide a highly effective and convenient antacid for poising gastric hyperacidity within this optimal range.

A PREVIOUS REPORT (1) presented a study of the rate of acid neutralization of a new antacid tablet, Creamalin tablet (Winthrop Laboratories), in comparison with ten similar antacid tablets. The new Creamalin tablet contains a specially prepared, highly reactive polymeric aluminum hydroxide hexitol complex. It was shown to have a much greater rate of acid neutralization than the other tablets tested. The present study was undertaken to evaluate the same antacid tablets from the additional standpoints of buffering range and duration of action.

It is felt that no single *in vitro* test method can furnish all the desired information regarding speed, duration, and buffering. Most of the previous work on this problem has employed the procedures of Johnson and Duncan (2), Rossett and Flexner (3), and various modifications thereof. In these procedures, acid is added to a given amount of antacid under physiological conditions and pH *versus* time recorded. The main difference in these methods is the manner in which acid is added to the reaction mixture. Some investigators add a single, large dose of acid while others employ the intermittent addition of small amounts. The method of Rossett and Flexner (3), and its subsequent refinement by Rossett and Rice (4), is reported to give results correlating very well with those obtained clinically. The latter state that the addition of acid at the rate of 240 ml. of 0.1 *N* hydrochloric acid per hour most nearly simulates the rate of secretion of the hyperchlorhydric stomach.

In order to interpret the data obtained by these methods, it is necessary to define the buffering range which will provide the most desirable antacid effect. The main objective of antacid therapy is the establishment of a level of gastric acidity that will permit healing and relieve pain with the least interference with essential digestive requirements. It is impossible to assign an absolute value for the most effective pH for this purpose; however, it is possible to select an effective range. Various pH ranges, 3.5–4.0 (2), 4.0–5.0 (5), 4.0–5.5 (6), and 4.0–6.0 (7), have been proposed, none of which seems to be entirely satisfactory. It is agreed that reduction in the free acidity of the stomach relieves the discomfort due to gastric hyperacidity, and it has been reported that relief may be achieved even at pH 2.5 (3). This seems probable considering the decrease in absolute acidity. Raising the pH of gastric juice (pH 1.5) to pH 2.5 or pH 3.0 represents a decrease in hydrogen ion concentration of 90 or 97 per cent, respectively. However, it seems safer to set pH 3.0 as the lower limit of the desirable buffering range.

Healing of peptic ulcers is facilitated by a decrease in peptic digestive activity. Peptic digestion is maximal at about pH 2.0 and decreases rapidly with increasing pH. All peptic activity ceases long before the stomach contents become neutral in a chemical sense. In fact pepsin is only very slightly active at pH 4.0 and inactive at pH 5.0. Kirsner (8) states that healing probably occurs at a pH lower than 4.0, indicating that complete abolition of peptic activity is not necessary. In addition, it is well established that alkalinizing the stomach is undesirable due to the possibility of acid rebound and alkalosis. Therefore, it appears unnecessary to raise the pH above 5.0 to control gastric hyperacidity and to permit healing of ulcers.

In view of the above facts it is evident that the

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main objective of antacid therapy can be met by maintaining the pH of the stomach contents within the range of pH 3.0–5.0. Since gastric hyperacidity is being very successfully managed with liquid aluminum hydroxide preparations, and since these exert their maximum buffering between pH 3.5 and 4.5, this still narrower range can be considered the optimal. The efficacy of an antacid, then, is directly dependent upon its ability to maintain the gastric contents within the prescribed ranges for prolonged periods of time.

EXPERIMENTAL

The materials studied were those employed in our prior investigation (1), namely, the new Creamalin tablet and ten leading aluminum hydroxide antacid tablets of comparable composition. This Creamalin tablet contains the new, highly reactive polymeric aluminum hydroxide hexitol complex in an amount equivalent to 320 mg. of aluminum hydroxide dried gel and 75 mg. of magnesium hydroxide. The other tablets all contain aluminum hydroxide as a major ingredient, alone or in combination with other antacid ingredients. Their compositions are given in Table I of our previous report (1), along with other pertinent data.

The procedure of Rossett and Rice (4) was modified by conducting the titrations at 37° in the presence of a small amount of wetting agent. The tablets were prepared by grinding to pass a 60 mesh screen. Aliquots of the powdered tablets were added to 70 ml. of distilled water in a constant temperature (37°) cell equipped with a mechanical stirrer and pH electrodes. The pH was tracked by a continuous electronic recorder. The pH electrode assembly in the cell consisted of a glass electrode and a salt bridge connecting to an outside calomel electrode according to the technique of Duggan and Stevens (9). Preliminary experiments showed that the calomel electrode became contaminated when placed in the reaction mixture.

Ten drops of a 10% nonionic surfactant (Triton-X-100) solution were added to insure wetting and dispersion since some tablets contain lubricants and other hydrophobic materials. At time zero, 30 ml. of 0.1 N HCl were added to the suspension, giving the equivalent of 100 ml. of 0.03 N HCl. Continuous addition of 0.1 N HCl was started immediately at the rate of 240 ml. per hour. The resulting pH changes were continuously plotted by the recorder. Triplicate runs were made, and the results averaged.

The dosages used for comparison were the same as those employed in our previous report (1). They were amounts of powdered tablets containing, (a) the average label dose, (b) 1.0 Gm. of active antacid ingredients, or (c) a total acid consuming capacity (U. S. P.) (10) of 250 ml. of 0.1 N HCl.

To confirm the desirable buffering range, titrations were carried out similarly on a liquid aluminum hydroxide gel of proved clinical effectiveness. Since the liquid had a total acid-neutralizing capacity (U. S. P.) (10) of 21.7 ml. of 0.1 N HCl per Gm., 11.52 Gm. samples (providing 250 ml. of 0.1 N HCl total acid neutralizing capacity) were included in the third comparison.

RESULTS

The titration curves of the products are given according to the three dosage comparisons described above. The titrations for most products were continued until the pH value dropped to pH 2.25 or below, at which point the antacid capacity can be considered practically exhausted. Since several products failed to raise the pH to this value, or exceeded it for only a short time, all titrations were continued for at least thirty minutes to show their general shape. The speed of pH change, buffering range maintained, and duration of antacid effect can be read from the curves presented in the figures.

Figures 1a and 1b show the results obtained for the tablets and the liquid aluminum hydroxide gel when the comparison was made on the equivalent acid neutralizing capacity basis. Note that the

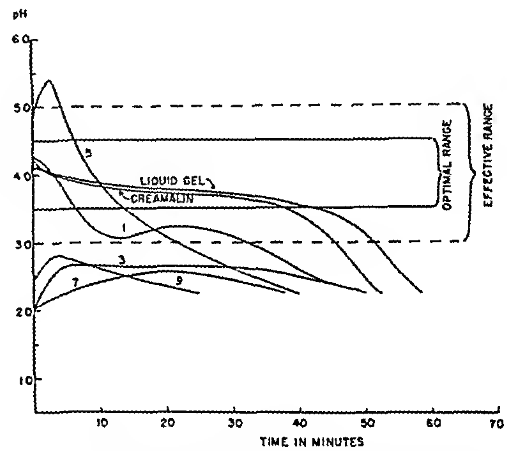

Fig. 1a.—Duration of action and buffering range at 37°. Samples equal weight of tablet material that will neutralize 250 ml. 0.1 N HCl by U. S. P. method. Start with 70 ml. water plus 30 ml. 0.1 N HCl and add 0.1 N HCl continuously at the rate of 240 ml. per hour.

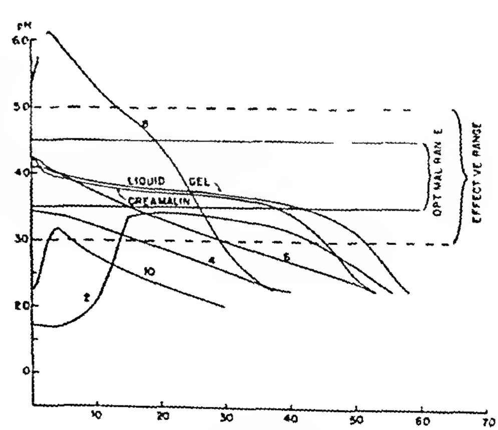

Fig. 1b.—Duration of action and buffering range at 37°. Samples equal weight of tablet material that will neutralize 250 ml. 0.1 N HCl by U. S. P. method. Start with 70 ml. water plus 30 ml. 0.1 N HCl and add 0.1 N HCl continuously at the rate of 240 ml. per hour.

liquid gel does indeed buffer within the optimal range, pH 3.5–4.5, and that approximately 85% of its effective duration of action is exerted therein. The curves for the new Creamalin tablet and the liquid are practically superimposable for their duration in the optimal range. This is due to the fact that the usual adverse effects of the drying and tableting operations on the activity of aluminum hydroxide gel have been largely eliminated in the new tablet. In contrast, only two other products, No. 1 (Fig. 1*a*) and No. 2 (Fig. 1*b*), exhibited any buffering in the broad effective range, pH 3.0–5.0. Three products, Nos. 3, 7, and 9 (Fig. 1*a*), showed buffering capacity but only at a level below the minimum effective limit. The others failed to show any significant buffering at all. It is also interesting to note the general shape of the curves for products Nos. 1 and 2. No. 1 shows an initial rapid fall for about the first ten minutes and then begins to exert its main buffering action. With No. 2 there is a latent period of fifteen minutes before this product begins its antacid activity. This would seem to indicate an undesirable delay in onset of action in a condition where rapid action is an essential requisite for prompt relief. Products No. 5 (Fig. 1*a*) and No. 8 (Fig. 1*b*) rapidly raise the pH above 5.0, but the pH then falls rapidly through the desired ranges.

In order to further quantitate the results, the times were measured during which the pH was maintained within the two ranges, i.e., the effective range, pH 3.0–5.0, and the optimal range, pH 3.5–4.5. These data are presented graphically in Fig. 2. The new Creamalin tablet held the pH in the broader range for forty-five minutes and in the optimal range for thirty-eight minutes. This represents 85% of the duration of action of the liquid gel in these ranges. It also has approximately 1¹/₄ and 2¹/₃ times as long a duration in the effective and optimal ranges as the next best tablets. Six of the tablets tested never brought the pH into the optimal range, and three of these did not even reach the lower effective limit.

The values obtained when using the average label dose as the basis of comparison are presented in Figs. 3*a* and 3*b*. It is quite evident from inspection that the new Creamalin tablet surpasses all of the others in its duration of action. The only other product which maintains the pH in the prescribed ranges for any length of time is No. 6, but its actual buffering power is meager as shown by the declining slope of the curve. Products No. 5 (Fig. 3*a*) and 8 (Fig.

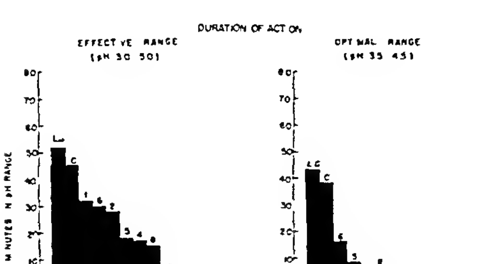

Fig. 2.—Duration of action in effective and optimal ranges. Samples equal weight of tablet material that will neutralize 250 ml. 0.1 N HCl by U. S. P. method. L G—liquid gel. C—Creamalin.

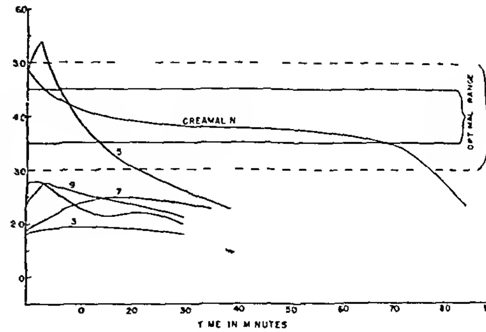

Fig. 3a.—Duration of action and buffering range at 37°. Samples equal weight of tablet material containing average label dose. Start with 70 ml. water plus 30 ml. 0.1 N HCl and add 0.1 N HCl continuously at the rate of 240 ml. per hour.

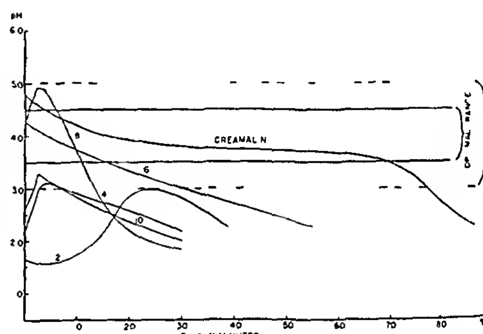

Fig. 3b.—Duration of action and buffering range at 37°. Samples equal weight of tablet material containing average label dose. Start with 70 ml. water plus 30 ml. 0.1 N HCl and add 0.1 N HCl continuously at the rate of 240 ml. per hour.

3*b*) drop quickly through these ranges after an initial rise to the upper limit. Product No. 2 (Fig. 3*b*) again shows a prolonged onset of action and reaches pH 3.0 for a very short interval. All the others buffer below the minimum acceptable level of pH 3.0.

The durations of action within the specified ranges for the average label doses are summarized in Fig. 4. The Creamalin tablet was active for sixty-five minutes in the optimal range and seventy-seven minutes in the effective range. This is 4 times and 2¹/₂ times the duration of the next best product in the respective ranges. Seven products never produced any effect in the optimal range, and three did not even bring the pH into the broader effective range. An important observation from this test is that the recommended average dosage is probably insufficient to achieve a satisfactory gastric environment in a majority of the products tested.

The comparison based on quantities of the antacid tablets which would contain 1.0 Gm. of active ingredients is presented in Figs. 5*a* and 5*b*. As with Figs. 1 and 3, the same general characteristics are apparent. Once more it can be seen that the Creamalin tablet produces the most prolonged effect. Product No. 2 (Fig. 5*b*) appears as second best, however, it again shows a delay of about fifteen minutes before pH 3.0 is reached. Products No. 5 (Fig. 5*a*) and 8 (Fig. 5*b*) raise the pH undesirably

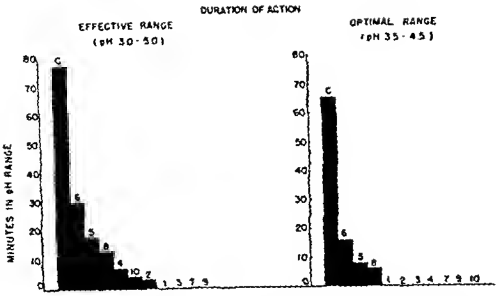

Fig. 4.—Duration of action in effective and optimal ranges. Samples equal weight of tablet material containing average label dose. C—Creamalin.

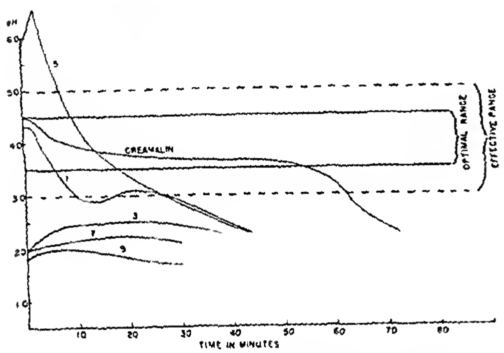

Fig. 5a.—Duration of action and buffering range at 37°. Samples equal weight of tablet material containing 1.0 Gm. antacid ingredients. Start with 70 ml. water plus 30 ml. 0.1 N HCl and add 0.1 N HCl continuously at the rate of 240 ml. per hour.

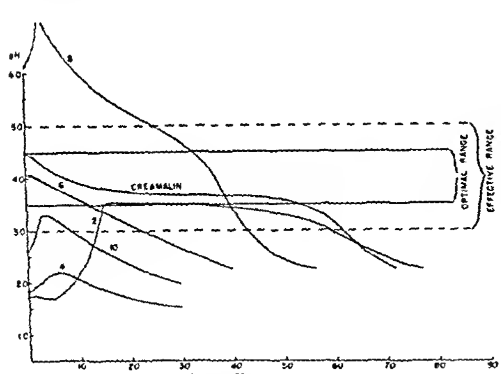

Fig. 5b.—Duration of action and buffering range at 37°. Samples equal weight of tablet material containing 1.0 Gm. antacid ingredients. Start with 70 ml. water plus 30 ml. 0.1 N HCl and add 0.1 N HCl continuously at the rate of 240 ml. per hour.

high at the start and do not show any prolonged buffering in the desired region. The curve for product No. 1 (Fig. 5a) also shows a characteristic dip to a point below pH 3.0 and only a short interval above this value. Product No. 6 (Fig. 5b) shows a steady decline indicative of limited buffering capacity. Product No. 10 held the pH above 3.0 for only seven minutes, whereas the remaining four products never reached this value.

The durations of action for this comparison are summarized in Fig. 6. The new Creamalin tablet maintains the pH within the optimal range for fifty-three minutes and within the effective range for sixty-two minutes. This is $2^1/_2$ and $1^1/_2$ times the duration, respectively, of the next best, product No. 2. Most of the other products show even less activity than can be accounted for by differences in the expected reactivity of their individual antacid ingredients.

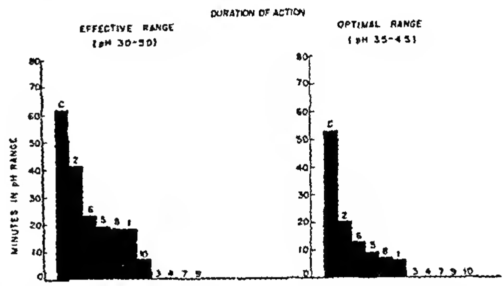

Fig. 6.—Duration of action in effective and optimal ranges. Samples equal weight of tablet material containing 1.0 Gm. antacid ingredients. C—Creamalin.

DISCUSSION

In the treatment of gastric hyperacidity the first concern of the patient is, of course, relief of discomfort. To be effective the antacid must have a rapid onset of action, i e., it must reduce the acidity of the gastric contents quickly to a pH range, pH 3.0 to 5.0 and preferably pH 3.5 to 4.5, which will give the desired relief. Although many alkalies will quickly reach this range, most of them overshoot and raise the pH too high. An ideal antacid should rapidly bring the pH to within these limits without exceeding pH 5.0, and preferably buffering between pH 3.5 and pH 4.5. The success of liquid aluminum hydroxide gel is probably due to the fact that it exerts its action in this manner. However, it is well known that some of the previously available aluminum hydroxide tablets have given disappointing results. The difference in reactivity between these tablets and the gel is considered to be of serious clinical importance (4). It is in order then to review briefly some of the factors responsible.

Contrary to the popular conception, so-called aluminum hydroxide has a very complex chemical composition and exists in polymeric forms with varying states of hydration. Aluminum hydroxide that is readily reactive with acid at body temperature represents a very delicate variation of this general structure. This is especially true with respect to the extent of hydration. Depending upon the nature of the original gel and the manner in which it is dried, it is possible to make dried gels which react very avidly with acid or are almost completely inert.

Another factor which is very important is the technique of manufacture of the tablet. Most tablets contain, in addition to the active ingredients, an assortment of diluents, binders, lubricants, disintegrants, and other materials requisite to the preparation of an elegant tablet. Variations in disintegration times and the presence of hydrophobic materials definitely affect the availability of the ac-

tive ingredients, even in chewing tablets. In compression of the tablet, the pressure exerted within the die, in the order of five tons, may create enough heat to further dehydrate the polymer and contribute to its loss of activity. The pressure may also make the tablet so hard that it becomes difficult to disperse and chew.

Most of these difficulties have been overcome with the development of the highly reactive polymeric aluminum hydroxide hexitol complex which has been incorporated into the new tablet. It has been demonstrated under physiological conditions that the buffering range and speed of reaction of this tablet are almost identical with that of a clinically effective liquid aluminum hydroxide gel and that its duration of action is only slightly less than a chemically equivalent quantity of the liquid. It also disperses readily even without chewing.

Another attribute of the new Creamalin tablet is apparent from an inspection of its titration curves. It buffers in almost exactly the same range regardless of the quantity used. An increase in dosage prolongs the duration of action without significantly shifting the resulting pH zone, illustrating the desirable balance between aluminum hydroxide and magnesium hydroxide that has been built into the tablet. This resistance to pH change with increased dosage is important when considering the frequency of administration to a patient with a highly active ulcer. There is little likelihood of alkalinizing the stomach through overdosing. This should allow the establishment of dosage schedules designed to overcompensate for variable gastric emptying time with the assurance that the pH of the stomach contents will remain in the desirable range.

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The Preparation of Some Amides of Bromal*

By WILLIAM D. EASTERLY, Jr., and VAN R. ROSA

A series of amides, including both aliphatic and aromatic compounds, were prepared for the purpose of evaluating their toxicity, antifungal activity, antispasmodic activity, and sedative-hypnotic effect.

A REVIEW of the work with various amide derivatives indicates that very little study has been made of the bromal amides. Therefore, in extension of the study of substituted amides (1), a number of amide derivatives of bromal have been prepared. Some of these are new compounds; others have been synthesized previously.

Bischoff reported the preparation of bromalurethane in 1874 (2), and Schiff and Tassinari prepared bromalacetamide about the same time (3). In 1932 the condensation of bromal hydrate with urea (4) was reported.

Yelburgi (5), in 1933, reported the chemical properties of a number of bromal amides, prepared by condensing bromal hydrate with a series of aliphatic amides from formamide to pelargonamide. Meldrum and Deohar (6) extended this work by condensing bromal hydrate with aromatic amides, including benzamide, phenylacetamide, and salicylamide. Chattaway and James (7) prepared the methylurea derivative of bromal.

More recently, bromal has been used in the preparation of compounds of the DDT type for insecticide studies. Cristol and Haller (8) used bromal in their production of 1,1,1-tribromo-2,2-bis (*p*-bromophenyl) ethane. Balaban and Sutcliffe (9) prepared aryl trihaloethanes by condensing two different benzene compounds such as chlorobenzene and fluorobenzene with bromal. Analogs of DDT have also been prepared by reacting bromal with aromatic ethers (10).

The preparation of some bromal amides for investigation was undertaken in view of previous work with various substituted amides. Byrum and LaRocca (11) showed the potentialities of amide derivatives as sedative-hypnotics in their investigation of certain chloral and α,α,β-tributyraldehyde amides. Anticonvulsant activity has been demonstrated for certain amides of dichloroacetaldehyde by Easterly and LaRocca (1). Other substituted amides, the α-bromoacetamides, have revealed remarkable levels of fungus inhibition (12).

EXPERIMENTAL

General. As no record has been found to date of their previous preparation, the phenylbutyramide

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Thanks are extended to Dr. J. P. LaRocca of the University of Georgia, School of Pharmacy, and to Dr. R. O. Bachmann of the University of Arkansas, School of Pharmacy, for their helpful suggestions.

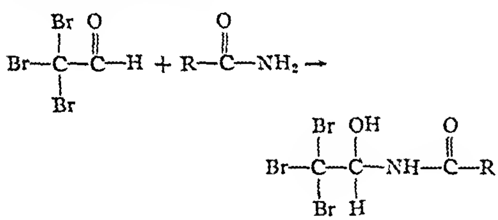
TABLE I.—BROMAL AMIDES[a]
$RCONHCH(OH)CBr_3$

Compound[b]	Yield, %	M. P. °C.[c]	Formula	Halogen Calcd. %	Halogen Found[d] %
Acetamide[e]	29	166	$C_4H_6Br_3NO_2$	70.8	72.2
α-Chloroacetamide	51	135	$C_4H_5Br_3ClNO_2$	73.5	72.1[f]
α-Phenylacetamide[g]	49	147	$C_{10}H_{10}Br_3NO_2$	57.7	56.2
Propionamide[h]	26	167[i]	$C_5H_8Br_3NO_2$	67.8	66.4
Butyramide[g]	30	146	$C_6H_{10}Br_3NO_2$	65.2	66.2
Isobutyramide[h]	33	154	$C_6H_{10}Br_3NO_2$	65.2	64.1
α-Phenylbutyramide	38	139	$C_{12}H_{14}Br_3NO_2$	54.1	53.5
Benzamide[g]	25	148	$C_9H_8Br_3NO_2$	59.7	58.8
Nicotinamide[j]	23	158	$C_8H_7Br_3N_2O_2$	59.6	59.6

[a] The "Chemical Abstracts" name for these compounds, N-(2,-2,-2-tribromo-1-hydroxyethyl) amides, was not used in this paper because it is unwieldy.
[b] All chemicals used were Eastman (Reagent) grade.
[c] Melting points taken on Fisher-Johns apparatus.
[d] Average of two determinations.
[e] Prepared previously by Schiff and Tassinari (3).
[f] The compound contains both chlorine and bromine, and the analysis is for total halogen.
[g] Prepared previously by Meldrum and Deohar (6).
[h] Prepared previously by Yelburgi (5).
[i] Melting point is lower than the 174° reported by Yelburgi (5).
[j] Reference (13).

and the chloroacetamide derivatives are reported as new, the preparation of the others has been reported (3, 5, 6, 13).

All the bromal amides reported in Table I were prepared by condensing bromal (tribromoacetaldehyde) with the appropriate amide. The general reaction equation is:



METHOD

Equimolar portions of bromal (tribromoacetaldehyde) and the appropriate amide were allowed to react, temperatures being maintained in a constant temperature bath. The optimum reacting temperature for the compounds prepared ranged from 21 to 37°. Condensation, indicated by the formation of a paraffin-like solid, varied from a few minutes for bromal nicotinamide to five days for bromal α-chloroacetamide. (The most suitable temperature and reacting time for the majority of the condensations was approximately twenty-four hours at 25°.)

The condensed masses were dissolved in hot alcohol, followed by purification and crystallization by essentially the same procedure as that previously reported for dichloroacetaldehyde derivatives (1).

Analytical Methods.—Each compound was first tested qualitatively for nitrogen and bromine by application of the sodium fusion process. Then quantitative determinations were made using the peroxide bomb fusion method for bromine.

As a check on procedure, a duplicate analysis for the bromine content of bromal acetamide was made by Oakwold Laboratories of Alexandria, Va.

The analytical data for the nine compounds are given in Table I.

SUMMARY

1. A satisfactory laboratory procedure for synthesizing some amide derivatives of bromal is described.

2. The physical properties of nine bromal amides are given.

3. Antifungal activity studies on the compounds have been conducted, with results to be published in another paper.

4. Additional pharmacological screening of the compounds is being carried out by The Upjohn Company, Kalamazoo, Mich.

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The Kinetics of Degradation of Chlorobutanol*

By A. DAMODARAN NAIR† and JOHN L. LACH

The degradation of chlorobutanol in aqueous solutions appears to be a specific hydroxide-ion-catalyzed reaction. The overall rate equation was found to be equal to k_1 [chlorobutanol] + k_2 [chlorobutanol] [OH^-]. The energy of activation and half-life periods were calculated. The half-life for the reaction in a buffered solution at pH 3 was calculated to be 90 years at 25° whereas the half-life under similar conditions at pH 7.5 was found to be 0.23 years.

CHLOROBUTANOL, a widely used bacteriostatic agent in pharmaceuticals, has been observed to undergo decomposition in solution. Ray and Basu (1) made solutions of chlorobutanol with and without sodium chloride and adrenaline hydrochloride, and determined the amount of chlorobutanol decomposed after autoclaving each solution in a cotton-plugged flask at 15 pounds pressure for thirty minutes. In addition to its loss due to volatilization, they observed that it is hydrolyzed to a considerable degree in solution after heating or on prolonged storage. van Esveld (2) stated that the adrenaline-chlorobutanol mixtures undergo no loss of potency even after storage for three months in ampuls. Gershenfeld (3) used chlorobutanol in dilutions of 1:200 to 1:1,000, and observed the antibacterial property against various organisms before and after heating the solutions for half-hour periods at 121, 100, and 65°. The results of his study showed that when heated, all solutions failed to show bacteriostatic properties against test organisms and spores used because of the decomposition of the preservative. He also observed that chlorobutanol decomposes readily in alkaline solution. Taub and Luckey (4) studied the effect of sterilization temperature, storage, and pH on the stability of solutions of chlorobutanol in ampuls. They noticed a drop in pH of the solution if the initial pH was above 4. They also observed that the decomposition is minimum between pH 3 to 6 in buffered solutions and between pH 3 and 4 in unbuffered solutions. Murphy, *et al.* (5), estimated the percentage of hydrolysis of aqueous chlorobutanol solutions at different hydrogen ion concentration after autoclaving at 121° for periods of time ranging from five to twenty minutes and states that the hydrolysis of chlorobutanol is slight at pH values below 5, but increases sharply in less acidic solutions. They also point out that when neutral or alkaline solutions of sufficiently high buffering capacity containing chlorobutanol are autoclaved the antibacterial effect is definitely impaired. Since the hydrolysis of chlorobutanol lowers the pH of unbuffered solutions the hydrolysis is therefore self-limiting.

Although a number of studies have been conducted on the stability of chlorobutanol in aqueous solutions, no attempt has been made quantitatively to study its decomposition. The purpose of this work, therefore, was to study the kinetics of degradation of chlorobutanol in aqueous solutions.

EXPERIMENTAL

Apparatus and Reagents.—Phosphoric acid buffers (0.4 *M*) of pH 2, 3, and 4; 0.4 *M* acetate buffers of pH 5 and 5.5; 0.4 *M* Phosphate buffers of pH 5.5, 6, 6.5, 7, and 7.5, constant temperature bath with Sargent heater, circulator, and regulator; thermometer calibrated to 0.1; Beckman pH meter, 5-cc ampuls, and apparatus and reagents used in "The Determination of Chlorobutanol in Pharmaceuticals by Amperometric Titration" (6), with the exception that 0.025 *N* NaCl solution and 0.02 *N* alcoholic $AgNO_3$ solution were used in the place of 0.08 *N* solutions of NaCl and $AgNO_3$.

Procedure Used for the Determination of pH and Temperature Effect.—One-tenth gram of chlorobutanol was accurately weighed into 250-ml volumetric flasks and solutions ranging in pH from 2 to 7.5 (in increments of 1 pH unit from pH 2 to 5 and of 0.5 unit from pH 5 to 7.5) were made by means of 0.4 *M* buffer solutions. About 3 cc of these solutions were transferred to 5-cc ampuls, sealed, placed in wire baskets, and immersed in constant temperature bath containing liquid paraffin. After a lapse of about three minutes to allow the solutions to equilibrate thermally, one ampul was taken out, chilled in cold water, and analyzed for ionic chloride content, as described in "The Determination of Chlorobutanol in Pharmaceuticals by Amperometric Titration" (6). This was repeated at definite intervals of time over a period of forty hours for solutions of pH 2 to 5.5 and for a shorter period of time for solutions of pH 6 to 7.5.

For solutions of pH below 6.5, measurements were made by setting the temperature of the bath at 93.5° and 85° and for solutions of pH above 6.5, measurements were made by setting the temperature of the bath at 65 and 75°. For solutions of pH 6.5, readings were taken at all the temperature settings mentioned above.

Initially, duplicate samples were withdrawn from the bath and analyzed, but this was later discontinued as a result of the degree of precision attained.

The equivalents of chloride ion found per liter of

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the chlorobutanol solution were calculated in the following manner:

$$\text{Equivalents of Cl}^-/\text{liter} = (\text{ml AgNO}_3)/2$$

where 2 is the volume of the sample withdrawn for analysis Since 3 Cl^- are liberated for every mole of chlorobutanol according to the stoichiometric equation

$$(CH_3)_2\text{—}C(OH)\text{—}CCl_3 \rightarrow 3\ Cl^-$$

the amount of chlorobutanol decomposed per liter of the solution can be calculated as follows:

$$\text{chlorobutanol moles/liter} = (\text{Eq. Cl}^-/\text{liter})/3$$

Procedure for the Determination of the Order of the Reaction with Respect to Chlorobutanol by Changing Its Initial Concentration.—Essentially the same procedure as above was carried out except for the necessary changes in the concentration of chlorobutanol The temperature was held at 75° and the pH was maintained at 6 5 The other concentrations of chlorobutanol solutions used were 0 08 and 0 12%.

Procedure for Determining the Effect of Change of Molarity of Buffer Solutions and also the Effect of Change of Ionic Species of Buffer Solutions.—Three-tenths molar and 0 2 *M* buffer solutions of pH 6 5 were made and the above procedure was repeated To note the effect of change of ionic species, 0 4 *M* phosphate buffer and 0 4 *M* acetate buffer, both of pH 5 5, were made and the rate of decomposition of chlorobutanol was studied

Characterization of the Degradation Products.—Chlorobutanol (0 25 mole) was decomposed with 1 mole of NaOH dissolved in 800 cc of distilled water (to obtain a 5% solution) at 5 to 10° Volatile components were removed by means of a steam bath The residual solution was acidified with phosphoric acid and steam-distilled, and 1,600 cc of distillate was collected

The above procedure was repeated using 5% alcoholic NaOH solution (the alcohol content being 90%). After removing alcohol and other volatile components on a steam bath, 800 cc of water was added to the residue and steam-distilled as before, and 1,600 cc of distillate was collected

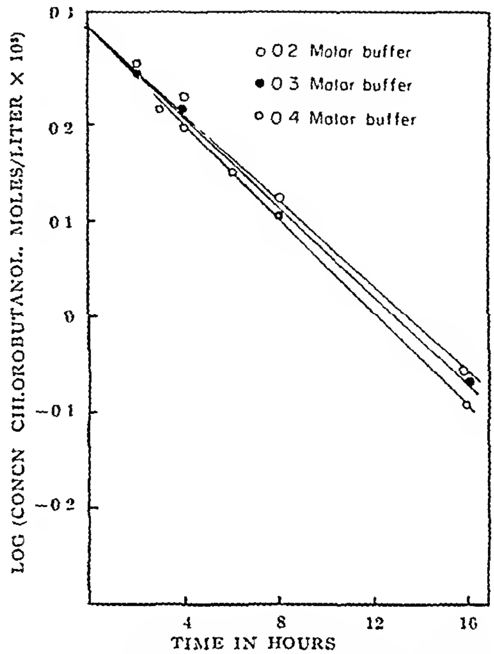


Fig 1—The effect of change of molarity of buffer on the rate of degradation of chlorobutanol at pH 6 5 and at 75°

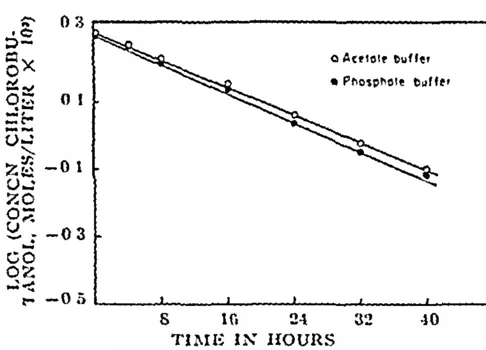


Fig 2—The effect of change of ionic species of buffers on the rate of degradation of chlorobutanol at pH 5 5 and at 85°.

RESULTS AND DISCUSSIONS

The results obtained indicate that the degradation of chlorobutanol in aqueous solutions is a base catalyzed reaction. Since there is no change in k, the specific reaction constant, when the reaction was carried out in buffer solutions of different molarity and of different ionic species, the hydrolysis appears to be a specific hydroxide-ion-catalyzed reaction (Figs. 1, 2)

The rate equation for the degradation may be written as follows:

$$\text{Rate} = k\, C_{\text{chlorobutanol}}{}^m\, C_{OH^-}{}^n$$

where k is the specific reaction constant Since the study was conducted in buffer solutions, OH^- concentration remained constant during each run and the rate equation is reduced to:

$$\text{Rate} = k'\, C_{\text{chlorobutanol}}{}^m$$

where $k' = K\, C_{OH^-}{}^n$.

The degradation is first order with respect to chlorobutanol, since a straight line relationship was obtained when the logarithm of the concentration of chlorobutanol was plotted against time at various pH, Figs 3–6 This can be further verified, Fig. 7, by plotting the amount of Cl^- produced during the hydrolysis against the various initial concentrations of chlorobutanol, since chlorobutanol when decomposed liberates chloride ions according to the stoichiometric equation:

$$(CH_3)_2\text{—}C(OH)\text{—}CCl_3 \rightarrow 3\ Cl^-$$

In Fig 8, the concentrations of Cl^- found after being held at 75° for sixteen hours at pH 6 5 have been plotted against the original concentrations of chlorobutanol.

It is obvious from the plot that the amount of Cl^- liberated is directly proportional to the initial concentration of the substrate, indicating the first order

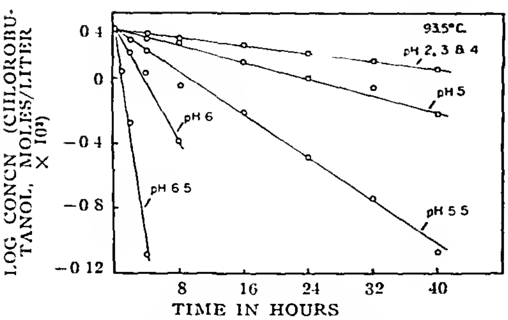


Fig 3—Log of the concentration of chlorobutanol against time in hours at 93 5°.

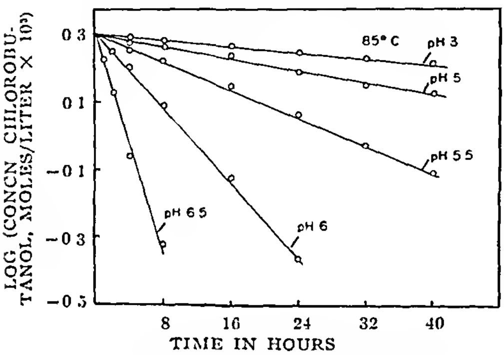


Fig 4.—Log of the concentration of chlorobutanol against time in hours at 85°.

dependency of the hydrolysis with respect to chlorobutanol

The order of the reaction with respect to OH^- may be obtained as follows:

Since

$$k' = K\, C_{OH^-}{}^{n} \qquad K = \text{constant}$$

$$\log k' = \log K + n \log C_{OH^-}$$

$$\log k' = \log K' + n\ \text{pH} \qquad K' = \text{constant}$$

$$\text{p}K_w = \text{pH} + \text{pOH}$$

Therefore a plot of log k' *vs.* pH should be a straight line, n, the order of the reaction, being equal to the slope of the line. Figure 9 represents the plot of log k' *vs.* pH at 93 5 and 85°. This plot shows that the reaction, with respect to OH^-, is zero order from pH 2 to 4 and is first order from pH 5.5 to 7.5

The overall rate may then be written as follows:

$$\text{rate} = \underbrace{k_1\, C_{(\text{chlorobutanol})}}_{1} + \underbrace{k_2\, C_{(\text{chlorobutanol})}\, (OH^-)}_{2}$$

Since the concentration of OH^- is very small at low pH, the second factor in the above rate equation becomes very small compared to the first factor and is negligible. Therefore, between pH 2 and 4, the rate is dependent on the concentration of chlorobutanol only, as indicated by the plot. From pH 5.5 to 7.5, the first factor is small and negligible when compared to the second factor in the above rate equation, and consequently the rate becomes dependent on the concentration of chlorobutanol as well as that of OH^-. Therefore, from pH 5 5 to 7.5, the rate is first order with respect to chlorobutanol and also first order with respect to OH^-, the overall reaction order being second order. Although this study was conducted up to pH 7 5, it

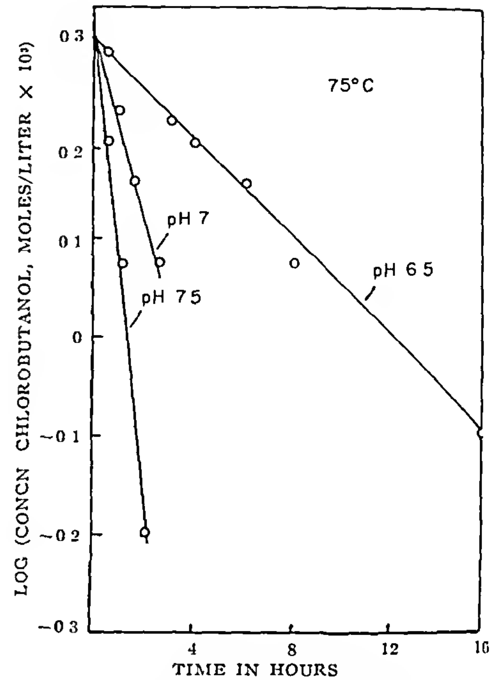


Fig. 5.—Log of the concentration of chlorobutanol against time in hours at 75°.

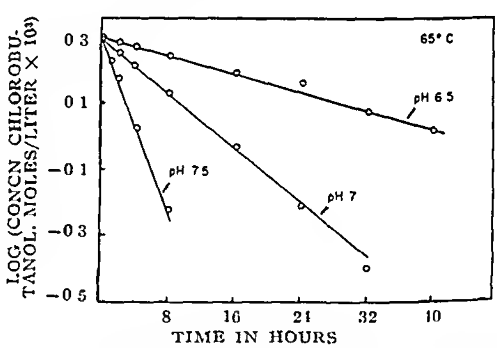


Fig. 6—Log of the concentration of chlorobutanol against time in hours at 65°.

would follow that the first order dependency on chlorobutanol would hold true for pH above 7.5 Between pH 4 and 5 5, the order of the overall reaction is not strictly first order or second order.

Specific reaction constants for the degradation of chlorobutanol in aqueous solutions at various pH were determined at four temperatures (Table I) and the Arrhenius type plot of log k' *vs.* $1/T$ was observed to give a straight line (Fig. 10). The apparent energy of activation, E_a, for these reactions at various pH's was determined from the slopes and found to be constant at 30 7 ± 0 8 kilocalories. Since this apparent energy of activation includes the heat of ionization of water (approximately 12 kilocalories), the energy of activation for the hydroxyl reaction would be 18 7 kilocalories, a reasonable value for hydrolytic reactions of this type.

The half-lives for chlorobutanol in aqueous solution at 25° were determined from the calculated

TABLE I.—THE SPECIFIC REACTION CONSTANT, k', FOR THE DEGRADATION OF CHLOROBUTANOL AT DIFFERENT pH AND AT DIFFERENT TEMPERATURES

	Temp., °C			
	65	75	85	93.5
pH	k', specific reaction constant, sec^{-1}			
2				3.95×10^{-6}
3			1.4×10^{-6}	4.06×10^{-6}
4				3.90×10^{-6}
5			2.86×10^{-6}	8.20×10^{-6}
5.5			6.74×10^{-6}	2.05×10^{-5}
6.0			1.81×10^{-5}	5.42×10^{-5}
6.5	4.43×10^{-6}	1.54×10^{-5}	5.98×10^{-5}	1.71×10^{-4}
7.0	1.36×10^{-5}	4.74×10^{-5}	1.66×10^{-4a}	4.42×10^{-4a}
7.5	4.17×10^{-5}	1.56×10^{-4}	5.29×10^{-4a}	1.42×10^{-3a}

[a] Calculated values from those experimentally obtained at 65 and 75°

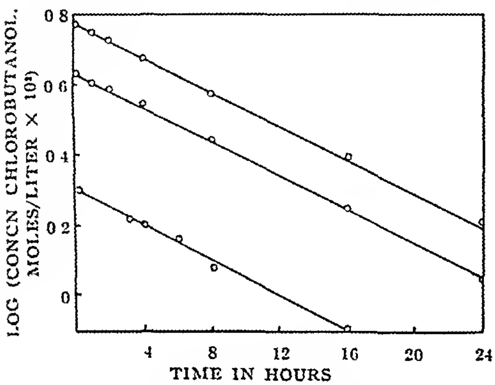


Fig. 7.—Log of the concentration of chlorobutanol against time in hours at pH 6.5 and at 75°. The plot shows the first order dependence of the reaction with respect to chlorobutanol, the rate of the reaction being directly proportional to the concentration of the substrate.

specific reaction constant, k' for reactions at various pH and at 25° (Table II, Fig. 11).

The Degradation Products.—The degradation products were qualitatively identified as acetone, carbon monoxide, chloride ion, and alpha hydroxyisobutyric acid. 2,4-Dinitrophenylhydrazine and *p*-nitrophenylhydrazine derivatives of acetone were prepared and the melting points were observed to be 126 and 152°, respectively. The presence of CO was detected by means of Saf-Co-meter[1] (carbon monoxide indicator). The chloride ion was detected with silver nitrate. The *p*-toluide and the *p*-nitrobenzyl ester of alpha hydroxyisobutyric acid were prepared and the melting points were observed to be 135–136° and 84–86°, respectively (which were 2 and 4° higher, respectively, from the reported ones). The amount of this acid obtained was very small in aqueous solutions: when chlorobutanol was decomposed with aqueous NaOH, acidified, and steam-distilled, the distillate was found to contain only 0.6% of acidic product whereas, when the same procedure was carried out in alcoholic NaOH, the distillate was found to contain 18% of acidic product. However, it has been observed by previous workers (7) that one of the methods of making alpha hydroxyisobutyric acid is by the action of alcoholic alkali on chlorobutanol.

The principal products of degradation of chlorobutanol in aqueous solution, consisting of chloride

[1] Saf-co-meter (carbon monoxide indicator). Developed by National Bureau of Standards, U. S. pat. 2,487,077.

TABLE II.—HALF-LIFE PERIODS, $t^1/_2$, FOR THE DEGRADATION OF CHLOROBUTANOL AT DIFFERENT pH AND AT 25°

pH	$t^1/_2$ (Years)	log $t^1/_2$
2.0	90[a]	1.9542
3.0	90	1.9542
4.0	90[a]	1.9542
5.0	40.3	1.6053
5.5	23.2	1.3655
6.0	9.0	0.9542
6.5	2.4	0.3802
7.0	0.61	−0.2147
7.5	0.23	−0.6383

[a] *Ea*, energy of activation, for the ... at pH 2 and 4 is the same as

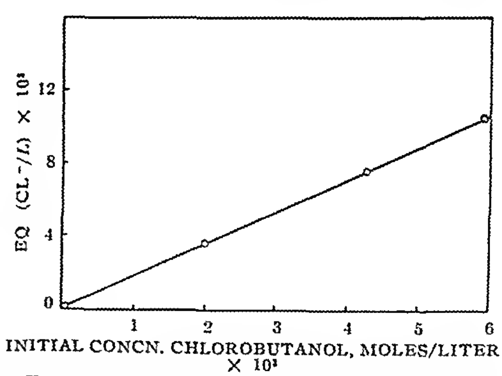


Fig. 8.—Influence of initial concentration of chlorobutanol on amount of chloride ion liberated at pH 6.5 and at 75°. The plot shows the first order dependence of Cl^- formation with respect to chlorobutanol.

ion, acetone, and carbon monoxide have been determined quantitatively by several investigators. On the basis of the stoichiometric relationship that each mole of chlorobutanol liberates three Cl^-, chlorobutanol has been quantitatively determined in this study. Sinton (8) and Jensen and Janke (9) have assayed chlorobutanol by determining acetone which is liberated on a mole to mole basis. Bressanin and Segre (10) have quantitatively determined the carbon monoxide liberated during decomposition of chlorobutanol.

The Mechanism of Degradation.—From the data obtained in the study of the degradation of chlorobutanol and the kinetics of its hydrolysis, some aspects of the mechanism of its degradation may be postulated.

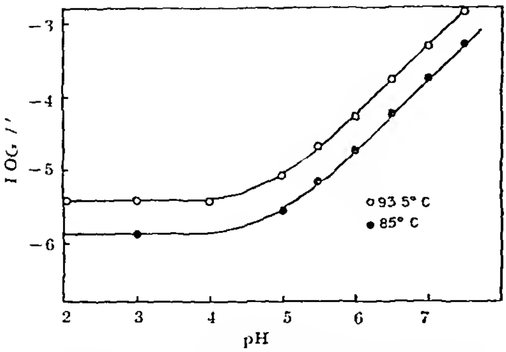


Fig 9—Log k', the specific reaction constant, against pH at 93 5 and 85°, showing the order of the reaction with respect to OH^- concentration

Ray and Basu (1) have suggested the following equations for the hydrolysis of chlorobutanol

$$(CH_3)_2{-}C(OH){-}CCl_3 \rightarrow (CH_3)_2{-}C(OH){-}COOH + 3\ HCl$$

$$(CH_3)_2{-}C(OH){-}COOH \rightarrow (CH_3)_2CO + CO_2 + H_2O$$

This mechanism does not appear to be correct since carbon dioxide was not identified as one of the decomposition products and since the above equations do not show the liberation of carbon monoxide which is apparently formed during the reaction

Bressanin and Segre (10) have suggested the following two types of mechanisms for the degradation of chlorobutanol

1 $(CH_3)_2{-}C(OH){-}CCl_3 \rightarrow (CH_3)_2{-}\underset{O}{C{-}C}{-}Cl_2 \rightarrow (CH_3)_2CO + CCl_2$

$$CCl_2 \xrightarrow{2OH^-} CO + 2Cl^- + H_2O$$

2 $(CH_3)_2{-}C(OH){-}CCl_3 \longrightarrow (CH_3)_2CO + CHCl_3$

$$CHCl_3 \longrightarrow CO + 3Cl^-$$

Hine (11), in his kinetic study of decomposition of $CHCl_3$ in 66²/₃% aqueous dioxane has proposed the following as the most likely mechanism for its decomposition

$$CHCl_3 \underset{}{\overset{\text{fast } OH^-}{\rightleftharpoons}} CCl_3^- + H_2O$$

$$CCl_3^- \xrightarrow[\text{slow}]{} CCl_2 + Cl^-$$

$$CCl_2 \xrightarrow[\text{fast}]{OH^-\ HOH} CO + HCO_2^-$$

However, chloroform is only very slowly decomposed in aqueous solution, and in order to effect complete decomposition of $CHCl_3$ it must be refluxed with NaOH for several hours (12) Therefore, it is evident from the kinetic study of the degradation of chlorobutanol liberating acetone, carbon monoxide, and chloride ion that, because the rate of degradation of chloroform in aqueous alkali is extremely slow when compared to that of chlorobutanol, chloroform is not an intermediate product during the hydrolysis of chlorobutanol

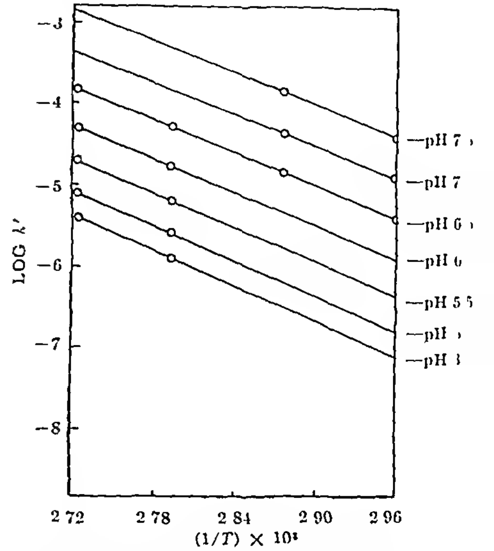


Fig 10—Arrhenius plots showing the temperature dependence of rate of degradation of chlorobutanol at different pH

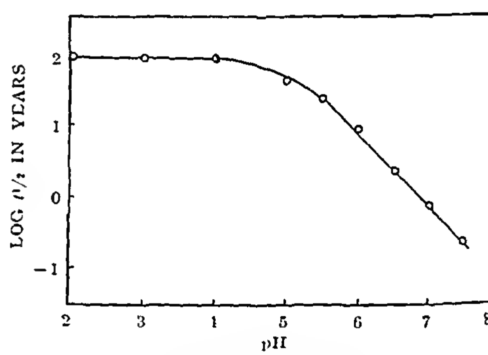


Fig 11—Log $t^{1/2}$, the half life period of the reaction, against pH at 25°

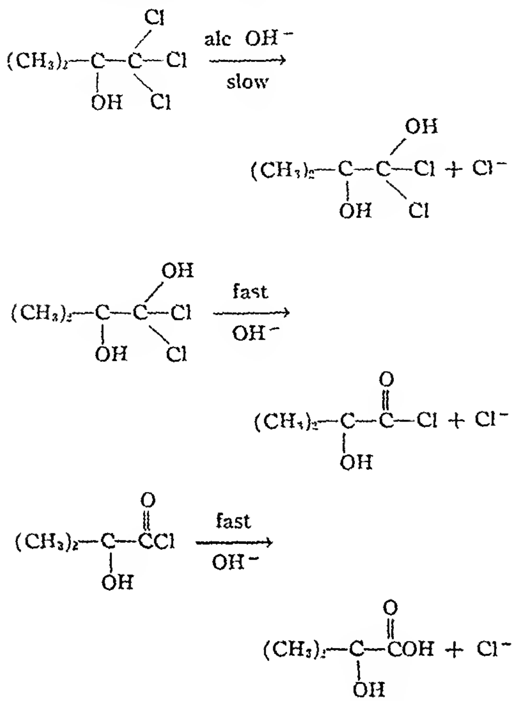
As has been pointed out, the degradation of chlorobutanol is independent of hydrogen ion concentration at low pH but dependent at pH of 5 and above It is unlikely that two separate mechanisms are involved in this decomposition The mechanism suggested by Bressanin and Segre (10) appears to be in accord with the available experimental data and is listed below

$$(CH_3)_2{-}C(OH){-}CCl_3 \xrightarrow[\text{proton transfer}]{OH^-\ \text{slow}} (CH_3)_2{-}\underset{O}{C{-}C}{-}Cl_2 + Cl^-$$

$$(CH_3)_2{-}\underset{O}{C{-}C}{-}Cl_2 \xrightarrow{\text{fast}} (CH_3)_2CO + CCl_2$$

$$CCl_2 \xrightarrow[\text{fast}]{OH^-\ HOH} CO + 2Cl^- + H_2O$$

Since α-hydroxyisobutyric acid was isolated in minute amounts from the degradation products, its formation is probably due to a competitive reaction as illustrated below:



This type of reaction seems to be highly favored in alcoholic medium (7).

SUMMARY AND CONCLUSIONS

1. The degradation of chlorobutanol in aqueous solutions appears to be a specific hydroxyl-ion-catalyzed reaction. It is first order with respect to chlorobutanol. With respect to OH^-, it is first order from pH 5 to 7.5 and zero order from pH 2 to 4. Although this study was conducted only up to pH 7.5, it would follow that the first order dependency on chlorobutanol would hold true for pH above 7.5.

2. The apparent energy of activation, E_a, for the degradation of chlorobutanol in aqueous solutions from pH 2 to 7.5 was found to be a constant at 30.7 ± 0.8 kilocalories. Since this apparent energy of activation includes the heat of ionization of water (approximately 12 kilocalories), the energy of activation for the hydroxyl reaction would be 18.7 kilocalories, a reasonable value for hydrolytic reactions of this type.

3. The half-lives at 25° for chlorobutanol in aqueous solutions were determined from the calculated specific reaction constants for reactions at various pH and at 25° The half-life of chlorobutanol in a buffered solution at pH 3 was calculated to be 90 years at 25° whereas its half-life under similar conditions at pH 7 5 was found to be 0 23 years Similarly, the decomposition of chlorobutanol in aqueous solution at 115° during a thirty minute heating period at pH 5 and at pH 6 were calculated to be 13 and 58 per cent, respectively.

4. It is to be noted that the loss of chlorobutanol from pharmaceuticals is due not only to chemical decomposition but also to the volatilization during preparation and during storage through porous containers, closures, etc.

5. The principal degradation products of chlorobutanol in aqueous solutions were found to be acetone, carbon monoxide, H^+, Cl^-, and a trace amount of α-hydroxyisobutyric acid.

6. Due to the production of H^+ during hydrolysis, the pH of the medium, if not buffered, is apt to be lowered. Because of this lowering of pH and because the rate of degradation is very slow at low pH, the hydrolysis would be somewhat self-limiting in unbuffered solutions.

7. While formulating chlorobutanol in pharmaceuticals, it is desirable to keep the pH of the medium at or below pH 4, since the rate of degradation of chlorobutanol is small and constant at pH 2, 3, and 4. If the pH of the medium is to be kept above pH 4, the amount of chlorobutanol that will be decomposed at that pH during preparation or storage at a particular temperature can be calculated from the data given in this paper and this calculated amount may be added in excess initially, wherever applicable, to compensate for the loss due to degradation.

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Studies on Local Anesthetics XVIII

Some New Quaternary Salts of Basic Carbamates

By ALEŠ SEKERA†, LIBOR NOVÁČEK, JAROSLAV SALAČ, and ČENĚK VRBA‡

Seven ethiodides and two ethochlorides of diethylaminoethyl esters of substituted carbamic acid were prepared and tested pharmacologically. The activity in both surface and infiltration anesthesia and acute toxicity tests was determined.

In most local anesthetics, e.g., substituted basic benzoates, anilides, acetophenones, arylethers, etc , the hydrophilic part of the molecule is represented by nitrogen whose tertiary form is to be preferred from the point of view of toxicity and local tolerance (substances with primary and secondary amine function are often less advantageous). Compounds including quaternary nitrogen have been neglected lately, on account, very likely, of the generally accepted rule that quaternization destroys anesthetic properties (1, 2)

It appears from studies on local anesthetic effects of compounds containing a quaternary ammonium group that quaternization of cocaine (3-5), procaine (5-8), Xyloeaine (7, 9), Stovaine (7), Pontocaine (7), and a whole series of other anesthetics (6, 7, 10-12) in most cases either reduces or destroys completely local anesthetic activity On the contrary, a few quarternary derivatives have shown (13-15) a rather high activity which proved to be several times higher than that of the corresponding tertiary bases (16, 17). There is thus a disagreement or even frequently the inconsistency between the results of the studies quoted. This might probably be explained by the variety of the pharmacological methods used, and above all, by the type of anesthesia used in tests, because the quaternary salts are generally more effective in both infiltration and conduction anesthesia than in surface anesthesia. The inconsistency between the results may also be explained by a retarded onset of anesthesia, so characteristic of these compounds, which might have prevented some workers from noticing their effect.

The mechanism of local anesthetic action of quaternary ammonium compounds is not yet known although some recent works (18) point out that the decomposition of a quaternary substance into a tertiary base is plausible and this conversion might aid in explaining the anesthetic action of the quaternaries, the tertiary base might constitute the very active form and its activity might be potentialized in the presence of the quaternary derivative. These phenomena might be associated with a depolarization of the nervous fibers or by slowing down of enzymatic metabolism. This mechanism appears to be supported by the fact that in both infiltration and conduction anesthesia, the activity of these substances is generally greater than in surface anesthesia; the type of anesthetic action (retarded onset, prolonged anesthesia with slow and irregular decrease) speak also in favor of this mechanism.

In order to determine the influence of quaternization on local anesthetic activity of basic esters of substituted carbamic acids, we prepared seven ethiodides (S 70–S 76) and two ethochlorides (S 84, S 85) of type I. These are quaternary compounds of seven basic carbamates (II)

$$\begin{matrix} R_1 \\ \diagdown \\ \end{matrix} N.COO.CH_2CH_2Y \quad \begin{matrix} \text{Type I: } Y = -\overset{+}{N}(C_2H_5)_3\,X^- \\ \text{Type II: } Y = -N(C_2H_5)_2 \end{matrix}$$
$$R_2 \diagup$$

chosen as characteristic representatives of the various structural types of compounds described in our previous papers (19-22). The structure of the prepared compounds are shown in Table I. The derivatives S 70 to S 72, whose structures already suggest their anesthetic inactivity, have been included in the studied series because of other pharmacological studies to be carried out.

The iodides S 70 to S 76 were prepared by heating the corresponding diethylaminoethyl esters (19-22) with the excess of ethyl chloride (method A); the chlorides S 85 and S 86 were prepared by a similar process by heating the tertiary basic esters with ethyl chloride in a sealed tube (method B) or from quaternary iodide by action of silver chloride (method C).

In order to carry out an improved investigation of the influence of quaternization on local anesthetic effect, we carried out in each case of active quaternary compound a simultaneous determination of the activity of the tertiary basic esters (S

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TABLE I.—QUATERNARY SALTS

$$\begin{matrix} R_1 \\ \quad\diagdown \\ \quad\; N\cdot COO\,CH_2CH_2\overset{+}{N}(C_2H_5)_3 \quad X^- \\ \quad\diagup \\ R_2 \end{matrix}$$

No	R_1	R_2	X^-	Method	Yield, %	M. P., °C	N Calcd., %	N Found, %	X Calcd., %	X Found, %
S 70	H	H	I	A	79	240[a]	8.86	8.64	40.14	40.36
S 71	C_2H_5	H	I	A	66	90	8.14	8.07	36.84	37.14
S 72	C_2H_5	C_2H_5	I	A	65	101	7.52	7.56	34.09	33.75
S 73	C_6H_5	H	I	A	73	131[b]	7.16	7.25	32.43	31.39
S 74	β-$C_{10}H_7$	H	I	A	75	195	6.33	6.49	28.69	28.57
S 75	p-$C_4H_9O\cdot C_6H_4$	H	I	A	86	97	6.03	5.98	27.33	27.25
S 76	C_6H_5	C_6H_5	I	A	62	228	5.98	6.20	27.09	26.70
S 84	β-$C_{10}H_7$	H	Cl	B	69	202	7.98	7.89	10.10	10.34
S 85	p-$C_4H_9O\cdot C_6H_4$	H	Cl	B	53	180	7.51	7.59	9.51	9.60
				C	75					

[a] Literature (28, 29) m. p. 205° and 245–246°, resp. [b] Literature (28) m. p. 128°.

TABLE II.—COMPARISON OF LOCAL ANESTHETIC EFFECTS AND OF TOXICITY OF QUATERNARY COMPOUNDS (I) WITH THE CORRESPONDING TERTIARY BASIC ESTERS (II)

Quaternary Compound (I) No.	Relative Activity: Surface Anesthesia[a]	Relative Activity: Infiltration Anesthesia[b]	LD_{50}, mg./Kg.	Corresponding Tertiary Basic Ester (II) No.	Relative Activity: Surface Anesthesia[a]	Relative Activity: Infiltration Anesthesia[b]	LD_{50}, mg./Kg.
S 73	Inactive[c]	(0.5–1)[d]	430	S 11	0.1	1.2	300
S 74	Inactive[e]	Inactive[e]	76	S 13	Irritation	1.3	610
S 75	Inactive[f]	Incomplete anesthesia[f]	150	S 46	1.4	3	430
S 76	Incomplete anesthesia	(1–2)[g]	(300)[h]	S 25	1.4	3.6	175
S 84	Irritation[i]	(1–2)[j]	110				
S 85	0.7	1.9	110				

[a] Cocaine = 1 (LD_{50} = 125 mg./Kg.). [b] Procaine = 1 (LD_{50} = 630 mg./Kg.). [c] Solution inactive at 2%, no effect on the tongue. [d] Irregular decrease of anesthesia with concentrations $M/5$ to $M/20$. [e] Experiment with saturated solution $M/100$. [f] Experiment with saturated solution $M/30$. [g] Irregular decrease of anesthesia with concentration $M/100$ (slight irritation). [h] Approximative determination (slight solubility 0.47%). [i] Concentrations between 1 and 5% cause irritation, a 1% solution is inactive. [j] Onset of anesthesia is retarded.

11, S 13, S 46, and S 25), already mentioned (19, 22) and studied by another pharmacological method (23).

EXPERIMENTAL[1]

Diethylaminoethyl Esters of Substituted Carbamic Acids.—These have been prepared by methods described in our previous communications (19–22) and isolated as bases. Esters derived from carbamic, ethyl carbamic, and diethylcarbamic acids have been purified by distillation under reduced pressure. The ester of 4-butoxycarbanilic acid has been recrystallized from petroleum ether. The other bases have been utilized as such.

Quaternary Salts.—*Method A.*—Two-hundredths of a mole of tertiary basic ester are refluxed for one and one-half hours with 15.6 Gm. (0.1 mole) of ethyl iodide. The excess of this was distilled under reduced pressure, the residue was dissolved in absolute alcohol and precipitated by anhydrous ether. The crystals formed were filtered off and crystallized in anhydrous alcohol-ether. In some cases the quaternary compounds precipitated as an oily mass which solidified on standing a few days.

[1] Melting points are not corrected. The analyses have been made in the analytical laboratories (director Dr. Ing. Z. Večeřa) of the Institute of Organic Syntheses, Pardubice-Rybitví, Czechoslovakia.

Method B.—One-hundredth of a mole of diethylaminoethyl ester of the substituted carbamic acid was heated for ten hours in a sealed tube at 80 to 100° with 26 Gm. (28 cc.; 0.4 mole) ethyl chloride. The product of the reaction was treated in the same way as in method A.

Method C.—A solution of 4.6 Gm. (0.01 mole) of substance S 75 (base) in 150 cc. water was shaken for five hours with 2.9 Gm. (0.02 mole) of freshly precipitated silver chloride. After filtration the solution was evaporated under reduced pressure and the residue treated as described in method A.

The yields, constants, and analyses are shown in Table I.

PHARMACOLOGY

The relative activity in surface anesthesia (rabbit cornea, $M/100$ cocaine as standard) and infiltration anesthesia (intradermal application to guinea pigs, $M/50$ procaine as standard) was calculated from the molar concentration, experimentally found to give the same effect as the standard. The method has been described in detail elsewhere (24, 25).

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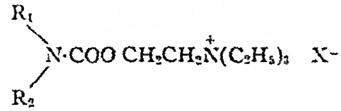

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DISCUSSION AND SUMMARY

By action of alkyl halides on diethylaminoethyl esters of substituted carbamic acids, nine corresponding quaternary salts have been prepared In this series the effect of quaternization on the activity in surface and infiltration anesthesia have been studied

The following correlation between molecular structure and pharmacodynamic action can be made

1 The nonsubstituted derivative S 70 and the aliphatic derivatives S 71 and S 72 are inactive as well as the corresponding tertiary basic esters S 10 (20), S 2 (21), and S 22 (20)

2 Aromatic derivatives (Table II) have been found active, although they showed very slight effects both in surface and infiltration anesthesia This fact is very probably increased or perhaps even caused by their low water solubility, the anesthetic activity of the chlorides S 85 and S 86, which are more soluble, is also higher

3 It appears from the comparison between the tertiary and quaternary esters that in this series quaternization lessens the activity in both surface and infiltration anesthesia

As far as the other pharmacodynamic effects are concerned, it has been found that these substances also have cholinergic (S 70–S 72), spasmolytic (S 75, S 76, S 85, and S 86), and high curare like activity (the effect of compounds S 73 to S 76, S 84, and S 85 are of the same order as the Flaxedil activity) The detailed results of these experiments will be published at a later date (27)

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Studies on Local Anesthetics XIX*

Substituted Phenylcarbamates of Piperidinopropanediol

By RUDOLF DOFEK, ALEŠ SEKERA†, and ČENĚK VRBA‡

The synthesis of twelve alkoxy- and two alkyl-substituted phenylcarbamates of piperidinopropanediol is reported. All the compounds are effective in both surface and infiltration anesthesia and are relatively nontoxic.

THE LOCAL ANESTHETIC effects of a number of arylcarbamates derived from basic propanediols has been studied in particular by Rider and his co-workers (1–4) Of this series, Diothane, 3-(1-piperidyl)-1,2-propanediol dicarbanilate hydrochloride has found practical application; the compound is also recorded in "New and Nonofficial Remedies" In the derivatives hitherto recorded, modifications have been carried out mainly in the basic component of the molecule, and the substitution of the aromatic nucleus of the carbanilate moiety has received relatively little attention

Working with series of diethylaminoethyl esters derived from substituted carbanilic (5–8) and diphenylcarbamic acids (9), we have found that methylation and particularly alkoxylation exerts a favorable effect on the pharmacodynamic ac-

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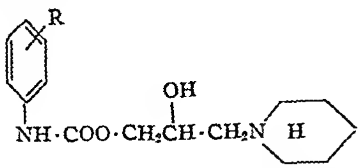
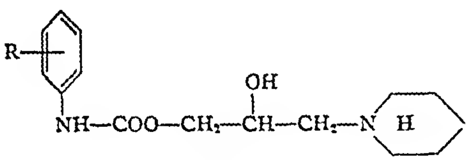
TABLE I.—SUBSTITUTED PIPERIDINOPROPANEDIOL MONOPHENYLCARBAMATE HYDROCHLORIDES



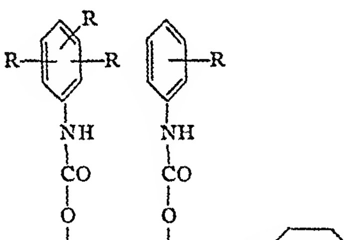
No	R	M p, °C	Solubility, H_2O, %	Yield %	N Calcd, %	N Found, %	Cl Calcd %	Cl Found, %
S 100	H	176[a]	50	91	8 90	8 99	11 26	11 11
(Monothane) picrate		172			13 80	13 70		
S 111	2-CH_3O	167–169	50	89	8 18	8 29	10 35	10 38
picrate		164–165			13 08	13 06		
S 112	2-C_2H_5O	144–145	(35–45)	90	7 80	7 77	9 88	9 82
picrate		138			12 70	12 72		
S 102	2-C_4H_9O	102	34	78	7 24	7 14	9 16	9 15
picrate		92–94			12 08	12 08		
S 103	3-C_4H_9O	137	(35–45)	93	7 24	7 14	9 16	9 04
picrate		126			12 08	12 09		
S 104[b]	4-C_4H_9O	175	35	88	7 24	7 26	9 16	9 07
picrate		108			12 08	11 88		

[a] Literature (1) m p 176–177°. [b] The crystalline base melts at 106 5° (ethanol) *Anal* —Calcd for $C_{19}H_{30}O_4N_2$: C, 65 11, H, 8 63, N, 7 99 Found: C, 65 00, H, 8 62, N, 7 95

tivity of compounds of this type. In an effort to extend these findings to esters of piperidinopropanediol we have now prepared a series of mono and diesters derived from this diol and alkyl- and alkoxy-substituted phenylcarbamic acids (Types I and II).

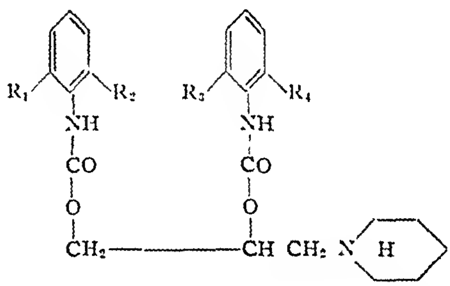


Type I



Type II

For the preparation of these compounds, the usual reaction of piperidinopropanediol with aromatic isocyanates was used. The use of solvent anhydrous ether as described by Rider did not prove suitable in our case because of the low reactivity of the substituted phenyl isocyanates; however, good yields of the products were obtained by refluxing the components in anhydrous benzene. Attempts to prepare diesters of types III to VI, derived from *o*-methyl or *o*-alkoxy-substituted isocyanates, were unsuccessful, presumably because of steric hindrance effects; only monosubstituted derivatives of the type I were isolated in each case.



Type	R_1	R_2	R_3	R_4
III	CH_3	CH_3	CH_3	CH_3
IV	OR	H	H	H
V	H	H	OR	H
VI	OR	H	OR	H

EXPERIMENTAL[1]

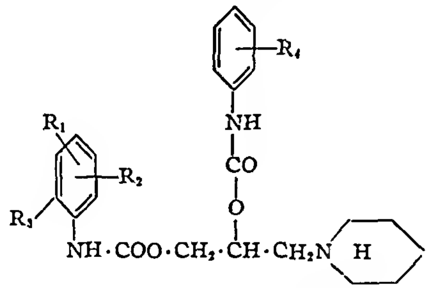
3-(1-Piperidyl)-propanediol-1,2.—This was prepared from 3-chloropropanediol-1,2 (10) and piperidine (11) and also from 2,3-epoxypropanol as an intermediate (12). Both methods under suitable conditions gave 50–60% yields but the second proved more reproducible, providing the temperature during the isolation of the epoxypropanol was kept as low as possible (b. p. *ca.* 30–60°).

Aromatic Isocyanates.—These were obtained by the method previously described (5–7), involving the reaction of the appropriate aromatic amines with excess of phosgene in boiling toluene. The yields and physical constants were as previously given (5–8).

Piperidinopropanediol Monophenylcarbamate Hydrochlorides (I).—To a boiling solution of 15.9

[1] All melting points are corrected and were obtained on the Kofler block. Microanalyses were carried out by Mrs. Klemova Parolková.

TABLE II.—SUBSTITUTED PIPERIDINOPROPANEDIOL BIS-PHENYLCARBAMATE HYDROCHLORIDES



No.	R_1	R_2	R_3	R_4	Method	M. p. °C.	Solubility H_2O %	Yield %	N Calcd. %	N Found %	Cl Calcd. %	Cl Found %
S 101	H	H	H	H	A							
(Diothane)						198[a]	1	92	9.68	9.56	8.17	8.20
picrate						118–120			13.11	13.31		
S 117[b]	2-CH_3	6-CH_3	H	H	B	200	0.8	95	9.09	9.08	7.67	7.63
picrate						202 dec.[c]			12.85	13.10		
S 118	2-CH_3	4-CH_3	6-CH_3	H	B	203	0.6	88	8.82	8.95	7.45	7.40
picrate						206[c]			12.58	12.69		
S 105	3-C_4H_9O	H	H	H	B	191–192	0.17	95	8.30	8.13	7.00	6.96
picrate						81–82			12.03	11.94		
S 114	H	H	H	3-CH_3O	C	109–110	3.6	90	9.05	8.97	7.64	7.56
picrate						168[f]			12.80	12.72		
S 116	H	H	H	3-C_2H_5O	C	125–127	1	95	8.79	8.79	7.41	7.38
picrate						181[d]			12.53	12.30		
S 106	H	H	H	3-C_4H_9O	C	134–136	0.3	91	8.30	8.30	7.00	7.09
picrate						174			12.03	11.89		
S 107	H	H	H	4-C_4H_9O	C	186	0.25	87	8.30	8.31	7.00	7.19
picrate						129			12.03	12.03		
S 109	3-C_4H_9O	H	H	3-C_4H_9O	A	153	0.16	88	7.26	7.38	6.13	6.28
picrate						126			10.90	10.81		
S 110	4-C_4H_9O	H	H	4-C_4H_9O	A	173	0.16	81	7.26	7.11	6.13	6.25
picrate						175			10.90	10.71		

[a] Literature (1) m. p. 197–198°. [b] The crystalline base melts at 137–138° (ether). *Anal.*—Calcd. for $C_{24}H_{31}O_4N_3$: C, 67.60; H, 7.30; N, 9.88. Found: C, 67.94; H, 7.13; N, 9.67. [c] From dioxane. [d] From acetone. [e] The crystalline base melts at 143° (ethanol). *Anal.*—Calcd. for $C_{30}H_{43}O_6N_3$: C, 66.51; H, 8.99; N, 7.75. Found: C, 67.21; H, 8.19; N, 7.69.

Gm. (0.1 mole) of piperidinopropanediol in anhydrous benzene, 0.1 mole of the appropriate isocyanate was added as a 20% solution in anhydrous benzene. The mixture was refluxed for thirty minutes, the benzene distilled off, the basic ester taken up in 500 cc. of anhydrous ether and precipitated as the hydrochloride by addition of one equivalent of ethereal hydrogen chloride. The crystalline hydrochlorides were washed with dry ether to neutral reaction (three to four portions of 100 cc. each). The hydrochlorides precipitated as oils were obtained crystalline by being kept *in vacuo* over P_2O_5 and KOH for several days, they were then ground and washed with anhydrous ether. The products were recrystallized from ethanol-ether after treatment with activated carbon.

The picrates were crystallized from 96% ethanol.

The melting points, yields, and analyses of the products are given in Table I.

Piperidinopropanediol Bisphenylcarbamate Hydrochlorides (II).—*Method A.*—Derivatives substituted in both benzene rings were prepared with 0.2 moles of the appropriate aryl isocyanate by the procedure used in the preparation of the monophenylcarbamates.

Method B.—Derivatives carrying a substituent in the phenylcarbamate residue attached to the primary alcoholic group of the piperidinopropanediol were prepared by treating 15.9 Gm. (0.1 mole) of piperidinopropanediol in 250 cc. of boiling anhydrous benzene with a 20% solution of 0.1 mole of the appropriate substituted phenylisocyanate in the same solvent, refluxing for thirty minutes, adding 11.9 Gm. (0.1 mole) of phenylisocyanate as a 20% solution in anhydrous benzene, refluxing for thirty minutes more, and working up as before.

Method C.—Derivatives carrying a substituent in the phenylcarbamate residue attached to the secondary hydroxyl group of the piperidinopropanediol were prepared as in *Method B*, except that the order of addition of the isocyanates was reversed.

The picrates were crystallized from 96% ethanol unless otherwise stated.

The methods of preparation, melting points, yields, and analyses of the products are given in Table II.

The solubilities of the hydrochlorides were determined by the mercurimetric titration of chloride ion in solutions saturated at room temperature. The results are given in Tables I and II.

PHARMACOLOGY[2]

The relative activity of the compounds in surface anesthesia (rabbit cornea, *M*/100 cocaine as stand-

[2] We are obliged to Miss S. Formačková for technical assistance with the pharmacological tests.

TABLE III —PHARMACOLOGICAL PROPERTIES

	Anesthetic Activity			Local Tolerance[a]			
				Probable Clinical Concn		Irritation Threshold	
No	Surface Anesthesia	Infiltration Anesthesia	LD_{50} mg /Kg	Conjunctiva % (anesth [b])	Subcutaneous %	Conjunctiva, %	Subcutaneous %
S 100	0.46	3.0	790	4 (∓) (60)	2+	4	1.5
S 101	2.9	4.8	890	1 (∓) (70)	1+	1	0.5
S 102	8.8	16.5	127	0.2 (∓) (60)	0.14∓	0.2	0.2
S 103	18	14	105	0.2 − (120)	0.1∓	0.4	0.1
S 104	5	6	430	0.2 − (60)	0.6 +	0.4	0.5
S 105	9.4	5–6	Spar[c] solub				
S 106	7.7	7.7	Spar[d] solub				
S 111	0.52	1.4	920				
S 112	1.2	2.7	690	4 − (45)	2 ++(+)	6	1
S 114	2.2	7.2	1,340	1 (∓) (60)	1.4 +	1	0.2
S 116	6.9	16.5	Spar[e] solub		0.28 (∓)		0.28
S 117	17	5.5	430	0.05 ∓ (35)	0.4 +	0.05	0.2
S 118	13	13.5	Spar[f] solub	0.5 (∓) (60)	0.5	0.5 +	50
Cocaine	1	3.6	125	5 − (40)	0.1 − 1	10	1–2
Procaine	0.15	1	630	10 − (30)	1 − 4	15	4

[a] − No irritation, + moderate irritation, ++ evident irritation +++ strong irritation [b] Duration of complete anesthesia (minutes) [c] $LD_0 \geqq 120$ (saturated solution) [d] $LD_0 \geqq 220$ (saturated solution) [e] $LD_0 \geqq 600$ (saturated solution) [f] $LD_0 \geqq 400$ (saturated solution)

ard) and infiltration anesthesia (intradermal application to guinea pigs, *M*/50 procaine as standard) was calculated from the molar concentration experimentally found to give the same effect as the standard. The method has been described in detail by Vrba and Sekera (13) and Roth (14).

The toxicity was studied according to Karber (15) by determining the LD_{50} in white mice (strain H) by subcutaneous application. The low solubility of some of the compounds did not permit the determination.

Local tolerance was studied in rabbits by application into the conjunctival sac and by subcutaneous injection at the root of the ear, and determining the threshold concentration causing local irritation. The tolerance of clinically probable doses was also studied. The results are given in Table III.

DISCUSSION AND SUMMARY

Sixteen derivatives of the Diothane series were prepared and tested for local anesthetic activities, toxicity, and irritation.

The following correlation between molecular structure and anesthetic activity can be made:

1. Ethoxylation and butoxylation of the benzene nucleus of carbanilic acid increases the activity of the esters of piperidinopropanediol. The same fact has already been observed previously in the case of simple basic esters (5–7, 9).

In the series of Diothane derivatives, less soluble in water than the derivatives of Monothane, the lipophilizaion of the molecule by monobutoxylation seems to be the limit; this appears for example from the results of tests of the slightly soluble substances S 107, S 109, and S 110, whose aqueous saturated solutions proved to be inactive.

2. Methoxylation is the least advantageous. In most cases the activity increases in proportion with the number of carbons of alkoxyl.

When the substitution of the benzene nucleus is considered, the *ortho* and *meta* positions are more advantageous than the *para*. These results agree as well with those obtained in our previous studies relating to simple basic carbamates (5–7).

3. Methylation of the benzene nucleus in the Diothane molecule highly increases the activity of surface and infiltration anesthesia.

In accordance with the pharmacological experiments which have been undertaken, several of the derivatives studied have proved to be more advantageous than the pattern substance, Diothane, especially from the point of view of surface anesthesia. Some of the more promising compounds are being tested clinically.

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Studies on Local Anesthetics XXI*

Some Derivatives of ω-Diethylaminoacetanilide

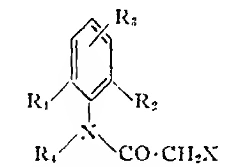
By ALOIS BOROVANSKÝ, ALEŠ SEKERA,† and ČENĚK VRBA‡

The preparation, surface, and infiltration activities and the toxicity of diethylaminoacetanilide and seven of its derivatives are described.

In our earlier publications we studied chiefly the basic esters of substituted carbamic acids By appropriate structural modifications we succeeded in preparing several very active, often slightly toxic compounds (1, 2). Several of these, in spite of their very favorable therapeutic index, proved themselves unsuitable for clinical trials because of their poor local tolerance (irritation)

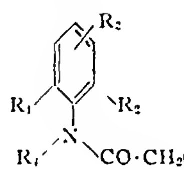
From the supposition that this undesirable property results chiefly from the formation of aromatic amine by the metabolism of the active carbanilate, we began studies of the substances, structures of which allowed us to suppose that the formation of aromatic amine would be decreased and, eventually, completely excluded In the choice of new structural types, in order to be able even in these new series to apply our knowledge of relations between the structures, the physico-chemical properties, and the anesthetic activity of the basic carbanilates, we have knowingly directed our attention to the compounds having structures similar to the substances previously studied. Our first work in that area was the preparation of basic alkoxy-propiophenones of the Falicaine series (3, 4) and of basic carbanilates (5, 6) of which the stability was augmented by (group —NH·COO—) the methylation of the benzene nucleus.

Continuing these studies, we decided to direct our attention to a closely related series—the basic anilides, on the supposition that the group Ar·NH·CO·R would be more slowly metabolized in the alkaline medium of human tissue than the Ar·NH·COO·R group. For the fundamental orientation in this new series, we prepared eight derivatives of type II; the description of the preparation and the results of the pharmacological experiments make the subject of this com-



Type I, X = Cl
Type II, X = $N(C_2H_5)_2$
R_1, R_3 = H, CH_3, C_4H_9O;
R_2 = H, CH_3, R_4 = H, C_6H_5

TABLE I.—SUBSTITUTED CHLOROACETANILIDES



No	R_1	R_2	R_3	R_4	Yield %	M p, °C
1	H	H	H	H	79	135[a]
2	CH_3	H	H	H	78	108–109[b]
3	CH_3	CH_3	H	H	79	142[c]
4	CH_3	CH_3	4-CH_3	H	74	172[d]
5	H	H	H	C_6H_5	90	119–121[e]
6	C_4H_9O	H	H	H	96	54[f]
7	H	H	3-C_4H_9O	H	82	94–95[g]
8	H	H	4-C_4H_9O	H	96	123–125[h]

[a] Dimroth (13) m p 131–135° [b] Literature (14) m p 111–112° [c] Literature (15) m p 145–146° [d] Literature (15) m p 178–179° [e] Literature (16) m p 118° [f] *Anal*—Calcd for $C_{12}H_{16}NO_2Cl$ (241 7) N, 5 80 Found N, 5 94 [g] *Anal*—Calcd for $C_{12}H_{16}NO_2Cl$ (241 7) N, 5 80 Found: N, 5 82 [h] Literature (9) m p 132 5°

munication Some substances have already been described in the literature (7–9) but nevertheless we have included them in our series in order to be able to study all together, utilizing the same pharmacological technique.

The structure of the substances studied can be seen in Table II. For their preparation we have utilized the method (8) starting from the corresponding aromatic amines which, passing through the chloroacetanilides (I), have supplied, in the reaction with diethylamine, the final basic anilides.

EXPERIMENTAL[1]

Mesidine.—This was prepared (5) by the catalytic hydrogenation of nitromesitylene over Raney nickel (*initial pressure 30 Atm*)

Butoxyanilines.—A similar mode of reduction was

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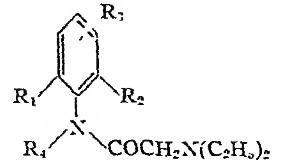
‡ Department of Pharmacology, School of Veterinary Medicine, Brno, Czechoslovakia

[1] All melting points were determined on a Kofler block and are corrected Microanalyses were carried out by Mrs Klemová Parolková

TABLE II.—SUBSTITUTED DIETHYLAMINOACETANILIDES



					Base			Hydrochloride	N		Cl	
No	R_1	R_2	R_3	R_4	Yield, %	b p., °C /mm	m p., °C	m p., °C	Calcd., %	Found, %	Calcd., %	Found, %
S 200	H	H	H	H	72	140–141/1 5[a]		108–109	11 54	11 38	14 61	14 39
S 201	CH_3	H	H	H	59	141 5/0 6[b]		120–122[c]	10 91	10 99	13 81	13 77
S 202	CH_3	CH_3	H	H	61	159/1[d]	70[d]	116–118[e]	9 70	9 62	12 28	12 24
S 203	CH_3	CH_3	4-CH_3	H	66	154–155 0 6[f]		140[g]	9 84	9 91	12 45	12 47
S 210	H	H	H	C_6H_5	88	175/1[h]		145–146[i]	8 79	8 49	11 12	11 36
S 220	C_4H_9O	H	H	H	56	182–183/0 7		118–120	8 90	8 80	11 26	11 14
S 221	H	H	3 C_4H_9O	H	45	186–187/0 5		123–125	8 90	9 10	11 26	11 14
S 222	H	H	4-C_4H_9O	H	53	185/0 5[j]	43–45	152–154[k]	8 90	8 78	11 26	11 10

[a] Literature (7) b. p 116–117°/0 15 mm [b] Literature (7) b p 159–160°/0 1 mm [c] From acetone [d] Literature (8) b p 159–160°/2 mm, m p 67°. [e] Sample for analysis dried over P_2O_5 by 110°/0 2 mm *Anal* —Calcd for $C_{14}H_{23}ON_2Cl \cdot H_2O$ (288 8) C, 58 22, H, 8 02 Found C, 58 51, H, 8 39 Even after prolonged drying under these conditions we were not able to prepare the anhydrous hydrochloride, described by Löfgren [f] Literature (8) b p 155–156°/0 6 mm [g] From acetone Sample for analysis dried over P_2O_5 by 110°/0 2 mm Literature (8) m p 136–137° [h] Literature (8) b p 194°/3 mm [i] *Anal* —Calcd for $C_{18}H_{23}ON_2Cl$ (318 9) C, 67 80, H, 7 27 Found C, 67 70, H, 7 31 [j] Literature (9) b p 186° 0 5 mm [k] Literature (9) m p 153°.

used in the preparation of these substances from the aromatic nitro-derivatives which were synthetized using the method described in one of our previous communications (1).

Chloroacetanilides (I).—In a solution of 0.3 mole of aromatic amine in 180 cc anhydrous acetone 60 Gm (excess *ca.* 80%) of the acid chloride of monochloroacetic acid was introduced in small portions The reaction completed, the mixture was, after one hour of settling, poured into 300 cc of hydrochloric acid (8%). The crystals of the chloroacetanilide were separated after twenty-four hours and washed free of acid.

The N-chloroacetyldiphenylamine was prepared from the diphenylamine and the chloride of the monochloroacetic acid in boiling toluene (8).

The samples for analysis were recrystallized from diluted alcohol The yields and constants of the prepared products are reported in Table I.

Diethylaminoacetanilides (II; S 200–S 222).—0 2 Mole of chloroacetanilides was refluxed for six hours with 37 Gm (0 5 mole) of diethylamine in 100 cc of anhydrous benzene The hydrochloride of diethylamine formed in the reaction was filtered off and the benzene solution washed twice with two 50-cc. portions of water. The solvent was removed by distillation. The residue was dissolved in 100 cc of hydrochloric acid (10%), and the solution extracted twice by 70 cc of ether. The acid solution was made alkaline by treatment with ammonia solution in order to liberate the base. This was extracted by ether (four times, 50 cc.) and the ethereal solution, dried on sodium carbonate, was fractionated.

The substance S 210 was prepared in anhydrous ether by heating for four hours (8)

The hydrochlorides were prepared in anhydrous ether. In the case of substance S 202 the isolation of a better defined crystalline base proved advantageous.

The yields, constants, and analyses of the prepared substances are collected in Table II.

PHARMACOLOGY[2]

The relative activity of the compounds in surface anesthesia (rabbit cornea, $M/100$ cocaine as standard) and infiltration anesthesia (intradermal application to guinea pigs, $M/50$ procaine as standard) was calculated from the molar concentration, experimentally found to give the same effect as the standard. The method has been described in detail by Vrba and Sekera (10) and Roth (11).

The toxicity was studied according to the method of Kärber (12) by determining the LD_{50} in white mice (strain H) by subcutaneous injection. The units are presented in Table III

DISCUSSION AND SUMMARY

The preparation and the results of the tests of surface and infiltration anesthesia and the toxic-

TABLE III.—PHARMACOLOGY OF SUBSTITUTED DIETHYLAMINOACETANILIDES

	Relative Activity		
Substance	Surface Anesthesia	Infiltration Anesthesia	LD_{50} s c mg Kg
S 200	Incomplete anesthesia[a]	0 12	890
S 201	<0 06[b]	0 24	450
S 202 (Xylocaine)	0 24	1 4	365
S 203 (Mesokain)	1	2 5	295
S 210	0 03	7 4	290
S 220	0 58	0 2	1,930
S 221	7 5	9	170
S 222	3	5 5	550
Cocaine	1	3 6	125
Procaine	0 15	1	630

[a] After application of $M/3$ solution [b] After application of $M/3$ solution complete anesthesia of 1 5 ± 2 minutes

[2] We are obliged to Miss M Perniková and I Růžicková for technical assistance with the pharmacological tests

ity of eight basic anilides of the Xylocaine series have been described The compounds have been shown to be effective in both surface and infiltration anesthesia; some of these have been found to be more active than the standards, namely, cocaine and procaine

The following correlation between molecular structure and pharmacodynamic action (Table III) can be made·

1 The methylation of the benzene nucleus of diethylaminoacetanilide in the *ortho* position increases the surface and infiltration anesthetic activities This augmentation of anesthetic effect can perhaps be explained, in addition to the influence of the physicochemical properties, by the increased stability of the anilidic group (Ar - NH CO R), which we have already mentioned in a previous communication (6)

2 As a result of the comparison of the effectuated tests of the activity and toxicity, it would seem that Mesokain (S 203) is more advantageous than Xylocaine (S 202)

3. Interesting also is the derivative S 210 which is seven times more active than procaine in surface anesthesia and is only twice more toxic Surprising is its slight activity in surface anesthesia

4. Butoxylation of the benzene nucleus of diethylaminoacetanilide increased surface and infiltration anesthetic activity in the series *ortho* < *para* < *meta* Parallel to this can be noticed an increase in the toxicity. The most advantageous therapeutic index is that of substance S 222 which is three times more active than cocaine in surface anesthesia, five and one-half times more active than procaine in infiltration anesthesia, and only slightly more toxic than procaine

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A Comparison of the Hypotensive Effects of the Monophosphoric Acid Ester of Thiamine and Thiamine Hydrochloride*

By NATHAN WATZMAN†, JOHN J. DeFEO‡, and JOSEPH P. BUCKLEY

The hypotensive effects of the monophosphoric acid ester of thiamine (RO 1-4788) and thiamine hydrochloride were investigated in anesthetized rats and dogs. The depressor effect of thiamine hydrochloride was 40.8% greater than that of RO 1-4788 in the rat and 42.8% greater than RO 1-4788 in the dog.

In recent years thiamine hydrochloride has created a new problem for the practicing physician. Clinicians (1–3) have reported that parenterally administered thiamine hydrochloride has produced peripheral circulatory collapse and a shock syndrome similar to that produced by vasodepressors and allergenic agents Reingold and Webb (4) reported a death following an intravenous administration of 100 mg. of thiamine hydrochloride Laboratory investigators have corroborated these findings in many species of animals (5–6) Mazzella (7) reported that 50 mg/Kg, i v, of thiamine hydrochloride produced arterial hypotension and bradycardia in the sympathectomized dog. These effects were still evident even after ganglionic blockade with tetraethylammonium chloride or adrenergic blockade with dibenamine indicating the possibility of a direct vascular depressant action Smith, *et al* (5), demonstrated, through perfusion of the mesenteric and femoral arteries of the dog and the isolated ear vein of the rabbit, that the vasodilatory property of thiamine hydrochloride was not necessarily a direct peripheral action but possibly central, involving depression of the vaso-

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constrictor centers. Wolfson and Ellis (8) reported that the vasodepressor effect of thiamine did not appear to be due to a direct action on blood vessels

French investigators (9) reported that the pyrimidine portion of the molecule was responsible for the visible toxic symptoms In a later paper (10) they suggested the possibility that excess thiamine produced too great a decarboxylation of pyruvic acid and that this disturbance in me abolism might be responsible for the resulting "thiamine shock" They reported a definite protection against the hypotensive activity of thiamine hydrochloride with 3 Gm /Kg sodium pyruvate, i v., in the rabbit

Since the monophosphoric acid ester of thiamine had been found to be as active as thiamine hydrochloride in vitamin B_1-deficient animals (11) it seemed desirable to compare the hypotensive and vascular effects of the two compounds (Fig 1)

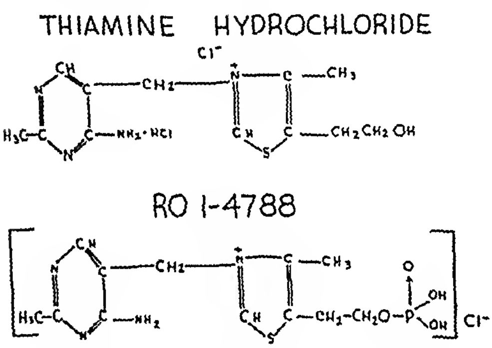

Fig. 1—Structural formulas of the compounds studied.

METHODS

Hypotensive Activity in Rats.—Wistar rats, weighing between 150 and 200 Gm , were anesthetized with 1 2 Gm /Kg of urethan intraperitoneally and direct carotid blood pressure recordings were obtained as previously described (12) Varying doses of thiamine hydrochloride (5-50 mg /Kg) were administered intravenously to a total of 26 rats to obtain the minimal dose producing a marked hypotensive response The selected dose of thiamine hydrochloride (20 mg /Kg) was administered intravenously to 30 rats (13 males, 17 females) and the equimolar dose (23 6 mg /Kg) of the monophosphoric acid ester of thiamine, henceforth designated as RO 1-4788, to another series of 30 rats (13 males, 17 females)

Hypotensive Activity in Dogs.—Mongrel dogs were anesthetized with pentobarbital sodium, 35 mg /Kg , administered intravenously The left carotid blood pressure was recorded with a mercury manometer or a Sanborn Electromanometer. The experiment was designed so that each animal served as its own control The test consisted of administering thiamine hydrochloride (10.0 mg /Kg) and RO 1-4788 (11.8 mg /Kg) alternately at three-hour intervals in a series of three doses to 20 dogs The sequence of dosage was reversed in 50% of the experiments Two per cent solutions of thiamine hydrochloride and RO 1-4788, having a pH of 3 3 and 3 1, respectively, were used In some of the experiments both derivatives were buffered to a pH of 7 with disodium phosphate

The effects of pretreatment with sodium pyruvate were investigated in three other dogs A dose of 5, 10, or 20 mg /Kg. of thiamine hydrochloride was administered via the femoral vein Sodium pyruvate, 1 Gm./Kg., was administered one hour after the test dose of thiamine, and two minutes later the test dose of thiamine hydrochloride was repeated

Peripheral Vascular Effects.—The effects of the intravenous administration of equimolar doses of the two compounds on the mesenteric vascular bed were studied in the rat meso-appendix preparation (13) The diameter of one of the magnified arterioles in the field was measured in mm units prior to and immediately after drug administration A total of 16 rats was utilized for this study.

RESULTS

Hypotensive Activity in Rats.—The effects of equimolar doses of thiamine hydrochloride and RO 1-4788 on the blood pressure of the rat are summarized in Table I The hypotensive activity of thiamine hydrochloride was 40 8% greater than that produced by the monophosphoric acid ester The difference between the responses was statistically significant at the 0 001 level when the data were subjected to the "Student" t test There was an apparent sex variation in response in that the difference between the mean responses in the males was highly significant ($P < 0\ 001$) whereas the difference in responses in the female rat was relatively insignificant ($P < 0\ 1$) The mean duration of action of thiamine hydrochloride and RO 1-4788 was one hundred and sixty-five and one hundred and seventy-seven seconds, respectively.

TABLE I.—THE EFFECTS OF THIAMINE HYDROCHLORIDE AND RO 1-4788 ON THE BLOOD PRESSURE OF THE RAT

		Drop in Blood Pressure, Mean %			
Sex	No	Thiamine HCl	RO 1-4788	t Test	P°
Mixed	60	31 1 ± 1 3	22 3 ± 1 6	4 3	<0 001
M	26	32 8 ± 2 03	19 4 ± 2 48	4 2	<0 001
F	34	29 8 ± 1 8	24 5 ± 2 1	1 96	<0 1

Mean ± standard ... of the mean
... between groups as determined

Hypotensive Activity in Dogs.—Thiamine hydrochloride elicited a hypotensive response in dogs which was 42 8% greater than that produced by RO 1-4788 (Figs 2 and 3, Table II) A variation in sex response was also noted in dogs since the ratio of the mean responses of RO 1-4788 to thiamine hydrochloride was 0 54 in males and 0 78 in females The buffered solutions of both compounds elicited the characteristic hypotensive effects observed with the unbuffered solutions

Pretreatment of the dogs with 1 Gm /Kg of sodium pyruvate partially blocked the hypotensive activity of thiamine hydrochloride The hypo-

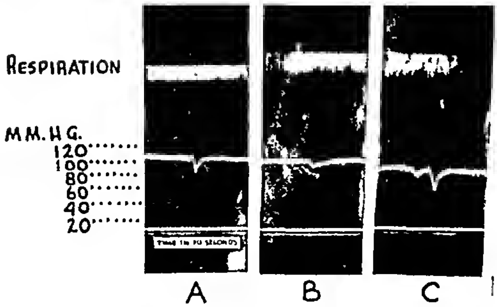

Fig 2 —The effects of thiamine hydrochloride and RO 1-4788 on the blood pressure of a normotensive dog, male, 5 8 Kg A and C effect of 10 0 mg /Kg of thiamine hydrochloride, B effect of 11 8 mg /Kg of RO 1-4788

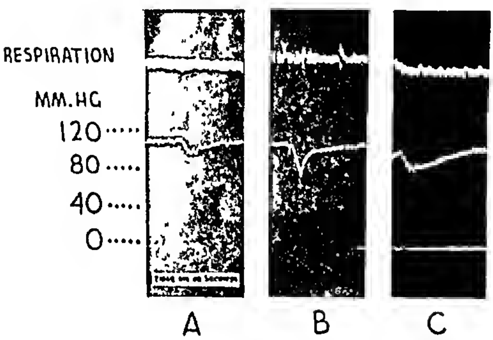

Fig 3 —The effects of thiamine hydrochloride and RO 1-4788 on the blood pressure of a normotensive dog, male, 9 07 Kg A and C—effect of 11 8 mg /Kg of RO 1-4788, B—effect of 10 0 mg /Kg of thiamine hydrochloride.

tensive activity of 5 mg /Kg was reduced 25 7%, 10 mg /Kg. reduced 53 0%, and 20 mg /Kg reduced 22 7%

Peripheral Vascular Effects.—Equimolar doses of the two thiamine derivatives (10 0 mg /Kg of thiamine hydrochloride and 11 8 mg /Kg of RO 1-4788) increased the size of the mesenteric vascular bed slightly in 8 of 16 preparations, however there was no apparent difference in the effects elicited by the two compounds

DISCUSSION

There was a very close similarity in the hypotensive activity of the compounds in rats and dogs The hypotensive activity of thiamine hydrochloride was 40 8% greater than RO 1-4788 in the rat and 42.8% greater than RO 1-4788 in the dog when equimolar doses were tested The variation in sex response was also similar in rats and dogs in that the difference in mean responses in the male was

TABLE II —THE COMPARATIVE EFFECTS OF THIAMINE HYDROCHLORIDE AND RO 1-4788 ON THE BLOOD PRESSURE OF NORMOTENSIVE DOGS

Male			Female		
Drop, Mean % Thiamine HCl	Drop, Mean % RO 1 4788	Ratio[a]	Drop, Mean % Thiamine HCl	Drop, Mean % RO 1-4788	Ratio[a]
14 2	6 1	0 48	9 4	10 3	1.10
9 7	4 9	0 51	27 1	5 2	0 19
29 3	7 0	0 24	19 4	9 1	0 47
17 2	25 4	0 54	12.6	12 5	0 99
17 7	20 6	1 16	10 3	8 5	0 83
23 1	7 2	0 31	16 8	11 4	0 68
22 2	12 3	0 55	11 0	11 8	1 07
		$\bar{X}$ 0 54	18 4	8.7	0.47
			13 4	12.0	0.90
			14 2	23 7	0 53
			10 4	17 0	1 72
			8 5	4 1	0 48
			13 7	10 5	0 76
					$\bar{X}$ 0 78

[a] RO 1 4788 thiamine HCl

much greater than the difference observed in females The difference in the mean responses in the male rat was statistically highly significant whereas the difference in means of the female was not significant at the 0 05 level ($P < 0.1, > 0.05$)

SUMMARY

1 The hypotensive activity of thiamine hydrochloride was 40 8 per cent greater than that of the monophosphoric acid ester of thiamine (RO 1-4788) in the Wistar rat

2 The hypotensive activity of thiamine hydrochloride was 42.8 per cent greater than the hypotensive response produced by RO 1-4788 in normotensive dogs.

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Separation of Decomposed Products of Phenobarbital Sodium by Paper Chromatographic Technique*

By A. J. KAPADIA, J. E. GOYAN, and J. AUTIAN

The decomposed products of phenobarbital sodium have been reported as consisting of phenylethylacetyl urea, phenylethyl acetic acid, and urea. Several solvent systems have been suggested for use in separating the various barbiturates from mixtures by paper chromatographic procedures. A paper chromatographic method has been developed to separate and identify the various decomposed products of phenobarbital sodium in aqueous solutions.

THE DEGRADATION of the barbiturates in alkaline solutions is well known to the pharmaceutical practitioner. One of the earliest references to the instability of malonyl urea derivatives was reported in 1903 (1). It was found that diethylacetyl urea was formed on heating barbital solutions. Husa and Jatul (2) have summarized the pathways of decomposition of the barbiturates in alkaline solutions. Rotondaro (3) has separated and identified the decomposition products of phenobarbital in alkaline solutions as being phenylethylacetyl urea which further hydrolyzes to produce phenylethyl acetic acid and urea. Fretwurst (4) studied the stability of seventeen barbiturates in alkaline solutions and quantitatively determined the decomposition products of the various drugs.

Even though chromatographic techniques have been employed for separating the various barbiturates from each other (5–9), the literature contains no information on the use of these techniques to separate the various decomposition products which may occur in barbiturate solutions. For this reason it was felt that a study should be initiated to ascertain if chromatographic procedures could be applied to effect a separation of the decomposed products of the barbiturates. Since the decomposition products of phenobarbital in alkaline solutions are known, this was the barbiturate selected to include in this study.

EXPERIMENTAL

Supplies.—Whatman filter paper No. 1; phenobarbital, U.S.P.; phenobarbital sodium, U.S.P.; phenylethylacetyl urea (prepared in our laboratory); phenylethyl acetic acid, A. R.; *n*-hexyl alcohol, *n*-heptyl alcohol, *n*-octyl alcohol, isopropyl alcohol, methyl alcohol, and ammonium hydroxide 28% (all A. R.); cobalt nitrate solution (1%); bromocresol green solution (0.04% in ethyl alcohol, adjusted to blue-green color with NaOH solution); *p*-dimethylaminobenzaldehyde solution (2% in 1.2 *N* HCl); copper sulfate solution (5% in 2 ml. pyridine and 48 ml. H_2O).

PREPARATION OF SAMPLES

A six per cent phenobarbital sodium solution was placed into ampuls and thermally degraded by storing at 80° for twenty-four hours or more. The ampuls were broken as required and the precipitate collected and dissolved in chloroform. Further extraction of the above aqueous phase with chloroform was then conducted to insure removal of all the chloroform-soluble constituents from the degraded phenobarbital sodium solutions. The chloroform and aqueous fractions were then collected into separate vessels and set aside until needed in the chromatographic studies.

Known decomposition products of phenobarbital sodium were obtained, recrystallized, and separately dissolved in either distilled water or chloroform.[1] At the same time an aqueous solution of phenobarbital sodium and a chloroform solution of phenobarbital were prepared.

Preparation of Chromatogram.—Approximately five microliters of each solution containing a known compound were then spotted on filter paper at equal intervals and at a starting point one and one-half inches from the bottom of the paper. These points appear on all the chromatograms (Figs. 1 and 2) at points A to F. At point G, spots were placed of all the known compounds (A–F) superimposed upon each other. The aqueous and chloroform fractions of the degraded phenobarbital sodium solution were spotted at points H and I, respectively. Finally, a mixture (H and I), spotted separately, of the aqueous and chloroform extracts of the degraded phenobarbital sodium solution was placed at point J.

The paper was then rolled into a cylindrical shape and placed into a battery jar containing a beaker or beakers of the desired solvent system. The lid was placed securely on the jar and the atmosphere in the chamber and the solvent system were allowed to equilibrate overnight, at which time the mobile phase of the solvent system was added to the bottom of the jar. After preliminary experimentation, it was found that eighteen hours were sufficient for the solvent front to reach the desired height. At the end of this period the chromatograms were removed and allowed to dry. It was then viewed in ultraviolet light. Phenobarbital sodium, phenylethylacetyl urea, and

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[1] Approximately 2% w/v solutions.

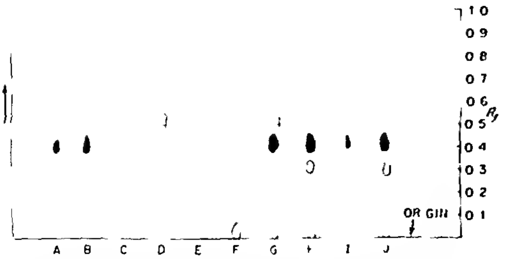

Fig 1 —Separation of the decomposition products of phenobarbital sodium by paper chromatography Solvent system isopropyl alcohol, 125 ml, n hexyl, or heptyl, or octyl alcohol, 75 ml, methanol, 50 ml, ammonium hydroxide, 50 ml

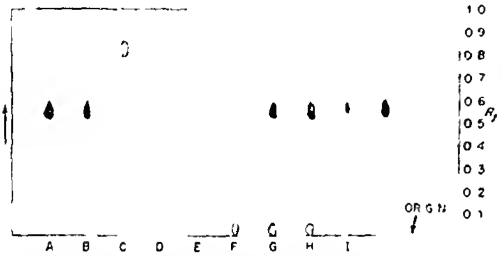

Fig 2 —Separation of the decomposition products of phenobarbital sodium by paper chromatography Solvent system n amyl alcohol saturated with borate buffer, pH 10

Key to the letters and symbols included in Figs 1 and 2 A—phenobarbital in chloroform, B—phenobarbital sodium in water, C—phenylethylacetylurea in chloroform D—phenylethyl acetic acid in chloroforom, E—urea in water F—sodium bicarbonate in water, G—mixture of A to F H—aqueous fraction of decomposed phenobarbital sodium solution I—chloroform fraction of decomposed phenobarbital sodium solution, J—mixture of H and I, solid area—phenobarbital and phenobarbital sodium enclosed vertical lines—phenylethylacetyl urea, enclosed horizontal lines—phenylethyl acetic acid, open area—urea enclosed dotted area—sodium bicarbonate

phenylethyl acetic acid were evident in the presence of the ultraviolet light Spraying techniques were also employed to delineate the various compounds on the chromatograms

Solvent System —In these studies two types of solvent systems were employed, a two phase and a one phase system

The two phase solvent system was prepared by shaking the solvents for five minutes in a separatory funnel and then allowing the phases to separate by standing for two hours at room temperature In this type of system, the following solvent combinations were employed n hexyl alcohol saturated with ammonium hydroxide (28%), n-hexyl alcohol saturated with borate buffer (pH 10), and n amyl alcohol saturated with borate buffer (pH 10)

When the buffers were employed, the filter papers were first sprayed with buffer solution and dried before they were spotted

For the one phase solvent system, the following combination of solvents was employed

isopropyl alcohol	125 ml
n hexyl alcohol or n heptyl alcohol or n octyl alcohol	75 ml
methyl alcohol	50 ml
ammonium hydroxide (28%)	50 ml

Spraying Reagents —The barbiturates were identified by spraying with cobalt nitrate solution, drying, and exposing the chromatogram to ammonia vapors Pink spots developed for the barbiturates Phenylethyl acetic acid appeared as a bluish green spot when sprayed with copper sulfate solution The solution of bromocresol green produced a yellow spot for the acidic compounds and a blue spot for the basic compounds The barbituric acids were not stained by this reagent because of their weakly acidic properties Urea was stained as a bright yellow color when sprayed with p dimethylaminobenzaldehyde solution

The spraying reagents did not stain any of the above compounds except urea when the filter paper was buffered, therefore, ultraviolet light was utilized for visualizing the compounds

RESULTS

Table I includes the R_f values obtained in the different solvent systems for the identified decomposed products of phenobarbital sodium Each solvent system is discussed below

***n*-Hexyl Alcohol Saturated With Ammonium Hydroxide System.**—When this solvent system was employed, it was possible to separate phenylethyl acetyl urea, phenylethyl acetic acid, and phenobarbital sodium from the decomposition mixture Urea was not completely separated from phenobarbital sodium Basic spots were found at the origin and were later identified as sodium bicarbonate with some trace impurities

***n*-Hexyl Alcohol Saturated With Borate Buffer System** —With this solvent system, it was possible to separate phenylethylacetyl urea, phenobarbital sodium, and phenylethyl acetic acid Urea, however, could not be separated from the phenylethyl acetic acid

Isopropyl Alcohol-Hexyl Alcohol-Methyl Alcohol-Ammonium Hydroxide System —In this one phase

Table I —Solvent System and R_f Values for Degradation Products of Phenobarbital Sodium

Name of Compound	n Hexyl Alcohol Saturated With Ammonium Hydroxide	n Hexyl Alcohol Saturated With Borate Buffer pH 10	Isopropyl Alcohol n Hexyl n Heptyl or n Octyl Alcohol Methyl Alcohol Ammonium Hydroxide	n Amyl Alcohol Saturated With Borate Buffer pH 10
Phenobarbital sodium	0 17	0 48	0 40	0 55
Phenylethylacetyl urea	0 90	0 85	0 81	0 83
Phenylethyl acetic acid	0 41	0 23	0 52	0 32
Urea	0 09	0 09	0 30	0 13

system (Fig. 1), a separation was achieved of the decomposition products in phenobarbital sodium solutions Spots for phenylethylacetylurea, phenylethyl acetic acid, phenobarbital sodium, and urea appear If the n-hexyl alcohol is replaced in the above system with n-heptyl or n-octyl alcohol no change will occur in R_f values (Table I) However, there appears a "tailing" of phenylethylacetic acid with the heptyl or octyl alcohols. The basic spot at the origin was identified as sodium bicarbonate.

Amyl Alcohol Saturated With Borate Buffer.—A very good separation of the four known decomposition products of phenobarbital (Fig 2) was obtained

SUMMARY

1. An ascending paper chromatographic technique was employed to separate the degradation products, reported by Rotondaro, of phenobarbital sodium in aqueous solutions.

2 Two solvent systems were found suitable to effect separation of the four decomposed products of phenobarbital sodium in an aqueous medium. One system employed n-amyl alcohol saturated with borate buffer while the second was a one phase system composed of isopropyl alcohol, n-hexyl alcohol, methyl alcohol, and ammonium hydroxide

3. Other degraded barbiturate solutions are being investigated by the method reported in this paper with the hope of separating and identifying the decomposed compounds.

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A New Benzodioxan Derivative With Adrenergic Blocking Activity*

2-(N,N-diallylcarbamylmethyl)aminomethyl-1,4-benzodioxan

By G. VICTOR ROSSI and MARVIN E. ROSENTHAL

The compound 2-(N,N-diallylcarbamylmethyl) aminomethyl-1,4-benzodioxan (A-2275), selected for evaluation from a series of new benzodioxan derivatives, produced moderate but rather transient reduction of blood pressure in anesthetized normotensive dogs and unanesthetized hypertensive rats. Its adrenergic blocking activity was less, and its direct vasoconstrictor effect was greater than that exhibited by piperoxan hydrochloride. Compound A-2275 possessed low acute toxicity and provided moderate protection against the lethal effects of epinephrine in mice.

A PREVIOUS REPORT by Rossi and Smith (1) described the evaluation of the hypotensive and adrenergic blocking activity of a new series of 2-[N-alkyl(N,N-dialkylcarbamylmethylamino) alkyl]-1,4-benzodioxan derivatives Synthesis and preliminary pharmacologic evaluation of additional members of this series indicated that 2-(N,N-diallylcarbamylmethyl)aminomethyl-1,4-benzodioxan (designated as compound A-2275) possessed significant depressor activity. The pharmacologic investigation of compound A-2275 forms the basis of the present report.

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This study was supported in part by a research grant from the National Drug Co, Philadelphia 44, Pa

EXPERIMENTAL

Acute Toxicity in Mice.—Swiss Webster male mice (Huntingdon Farms), weighing from 18 to 22 Gm. and fasted for twelve hours with free access to water, were used in determining the oral, intraperitoneal, and intravenous LD_{50} values of compound A-2275[1] and piperoxan hydrochloride (Benodaine).[2] Following preliminary "range-finding" experiments, each LD_{50} was established from data obtained with five groups of ten mice which received dosages spaced at 0.1 log intervals. Those animals which succumbed, did so within two hours after administration of the benzodioxan derivatives, and in each case death was preceded by hyperexcitability, tremor, clonic and tonic convulsions. LD_{50} values and their 95% con-

[1] Generously supplied by Dr. Gustav J. Martin, National Drug Co. Research Laboratories, Philadelphia, Pa.
[2] Generously supplied by Edmund McLaughlin, Merck Sharp and Dohme, West Point, Pa.

fidence limits, calculated according to the method of Epstein and Churchman (2) are presented in Table I

TABLE I —ACUTE TOXICITY OF PIPEROXAN HYDROCHLORIDE AND COMPOUND A 2275 IN MICE

	LD_{50} mg /Kg	95% Confidence Limits
Piperoxan hydrochloride		
Intravenous	33 2	30 2 – 36 1
Intraperitoneal	175 0	159 0 – 189 0
Oral	502 0	427 0–587 0
Compound A 2275		
Intravenous	55 0	50 2– 60 1
Intraperitoneal	230 0	209 0–252 0
Oral	447 0	392 0–509 0

The data do not demonstrate any outstanding difference in acute toxicity between piperoxan hydrochloride and compound A 2275, however, the ratios between the intravenous and oral LD_{50}'s suggest that the latter compound may be absorbed to a greater extent following oral administration

Reduction of Epinephrine Toxicity in Mice.—The degree and duration of protection provided by compound A 2275, piperoxan hydrochloride, and tolazoline hydrochloride (Priscoline) were compared in mice (Swiss Webster male, 18–22 Gm , fasted twelve hours) according to the method of Loew and Micetich (3) Varying doses of the three adrenergic blocking agents or 0 5 ml of distilled water were injected intraperitoneally into a number of groups of ten mice At intervals of fifteen, thirty, sixty, one hundred and twenty, and two hundred and forty minutes after administration of either distilled water or the test compounds, 14 4 mg of *l* epinephrine hydrochloride was injected intraperitoneally into groups of pretreated mice and mortality occurring after one hour was recorded (Table II)

The dose of epinephrine employed in this study killed an average of 86% (N=50) of the control animals (previously injected intraperitoneally with 0 5 ml of distilled water) Piperoxan hydrochloride in doses of 50 and 100 mg /Kg failed to confer protection against the lethal effect of epinephrine Loew and Micetich (3) similarly reported piperoxan to be noneffective in this respect Administration of epinephrine fifteen minutes after intraperitoneal injection of 50 mg /Kg of compound A-2275 resulted in fewer fatalities than in the control group Maximal protection (50% mortality) occurred within thirty minutes, and after one hour the blocking action had largely terminated Only slightly greater reduction of epinephrine toxicity was obtained with 100 mg /Kg of compound A-2275 A considerably greater degree and duration of protection against the toxic effect of epinephrine was provided by significantly smaller doses (10 and 20 mg /Kg) of tolazoline

Although the benzodioxan compound A 2275 moderately decreased the toxicity of epinephrine in mice, the doses required to elicit protection were considerably in excess of those producing significant depressor activity as described in subsequent sections of this report

Hypotensive Activity in Normotensive Dogs—Compound A 2275 and piperoxan hydrochloride were compared in regard to their ability to lower blood pressure and reduce the pressor response to epinephrine and occlusion of the carotid arteries in mongrel dogs (8 to 10 Kg , both sexes) anesthetized with 35 mg /Kg of pentobarbital sodium Blood pressure was recorded directly from a femoral artery Epinephrine hydrochloride (2 μg /Kg) was injected intravenously (femoral vein) thirty minutes after administration of the compound being examined After stabilization of the blood pressure, the carotid sinus reflex was evoked by clamping both common carotid arteries for an interval of thirty seconds The changes in blood pressure following each procedure were compared with the average of three uniform pressor responses elicited prior to administration of the hypotensive agent The mean reduction in blood pressure and inhibition of the pressor effect of epinephrine and carotid artery occlusion obtained with each dose level and route of administration are presented in Table III

Compound A-2275 injected intravenously in a dose of 1 mg /Kg produced a moderate, rapid but transient (fifteen to thirty minutes duration) reduction in blood pressure and the response to epinephrine and carotid artery occlusion Increased intravenous doses (as much as 16 mg /Kg) did not elicit appreciably greater decreases in blood pressure, but the duration of hypotension was increased from sixty to one hundred and eighty minutes Doses of 32 mg /Kg intravenously were uniformly fatal The partial adrenergic blockade produced by even larger doses of compound A 2275 was abolished by increasing the amount of epinephrine injected to 4 or 8 μg /Kg Similar blockade of the pressor response to norepinephrine (2 μg /Kg) was observed, however the effects of intravenous isopropylarterenol (2 μg /Kg) were not significantly altered

The reduction of blood pressure following oral administration of 25 mg /Kg of compound A-2275 was maximal within forty-five minutes and was largely terminated after three hours Increasing the oral dosage to 50 mg /Kg increased the onset and duration of action (four hours) but did not ap-

TABLE II —REDUCTION OF EPINEPHRINE TOXICITY IN MICE

Test Compound	mg /Kg i p	Per Cent Mortality[a] — Minutes after Administration of Test Compound 15	30	60	120	240
Tolazoline hydrochloride	10	10[b]	20	60	70	90
	20	10	10	40	50	80
Piperoxan hydrochloride	50	80	90	100	90	80
	100	90	100	100	100	90
Compound A-2275	50	60	50	80	80	90
	100	50	50	70	90	100
Distilled water	0 5 ml / mouse	80	100	80	80	90

[a] Deaths recorded one hour after injection of epinephrine hydrochloride—14 4 mg /Kg , i p [b] N = 10 in all groups

TABLE III.—DEPRESSOR ACTIVITY OF COMPOUND A-2275 AND PIPEROXAN IN THE ANESTHETIZED NORMOTENSIVE DOG

Compound	Dose mg./Kg.	Route of Administration	No. Animals	Mean Per Cent Decrease in Initial Blood Pressure	Mean Per Cent Decrease in Pressor Response 30 min. after Drug: Epinephrine[a]	Carotid Sinus Occlusion[b]
A-2275	1	i. v.	5	20	16	34
A-2275	2	i. v.	7	23	18	42
A-2275	4	i. v.	5	26	38	48
A-2275	8	i. v.	5	24	45	52
A-2275	16	i. v.	4	26	62	65
A-2275	25	*per os*	4	28	40	72
A-2275	50	*per os*	5	38	42	80
Piperoxan	0.25	i. v.	1	0	100	0
Piperoxan	1	i. v.	2	0	reversal	26
Piperoxan	2	i. v.	1	16	reversal	85

[a] Epinephrine = 2 μg./Kg. of epinephrine hydrochloride, intravenously.
[b] Carotid sinus occlusion = clamping both common carotid arteries for thirty seconds.
Per cent decrease in pressor response based on an average of three responses prior to administration of compound A-2275 or piperoxan.

TABLE IV.—COMPARATIVE HYPOTENSIVE EFFECT OF COMPOUND A-2275 AND PIPEROXAN IN THE UNANESTHETIZED HYPERTENSIVE RAT

Compound	Dose mg./Kg.	Route of Administration	Mean Pretreatment Systolic Blood Pressure mm. Hg	Mean Reduction of Pretreatment Blood Pressure mm. Hg, Time After Administration of Drug, Hr.: 1	2	4	6
A-2275	1	i. p.	218[a]	18	19	8	2
Piperoxan	1	i. p.	222	18	22	11	2
A-2275	2	i. p.	231	23	31	19	3
Piperoxan	2	i. p.	226	15	27	16	0
A-2275	4	*per os*	230	18	20	11	1
Piperoxan	4	*per os*	215	15	20	10	1
A-2275	8	*per os*	207	24	29	14	2
Piperoxan	8	*per os*	212	21	27	14	2

[a] N = 10 in all groups.

preciably enhance the extent of the blood pressure reduction.

That mechanisms other than adrenergic blockade contribute to the hypotensive activity of compound A-2275 was suggested by the partial inhibition of the pressor responses to epinephrine and norepinephrine obtained in the dog with doses of the compound which produced considerable reduction of blood pressure. Piperoxan abolished or reversed the pressor response to epinephrine in concentrations which elicited no significant effect on blood pressure.

Hypotensive Activity in Hypertensive Rats.[3]—Chronic hypertension was experimentally induced in male Sherman rats weighing approximately 130 Gm. according to the technique previously described by Rossi and Packman (4). Systolic blood pressure was determined in the unanesthetized hypertensive rat by the photoelectric tensometer method (5). The hypotensive activity of compound A-2275 in doses of 1 and 2 mg./Kg. injected intraperitoneally, and 4 and 8 mg./Kg. given orally, was compared in each case with piperoxan hydrochloride administered in the same doses.

No measurable change in the blood pressures of ten control animals was detected when measured one, two, four, and six hours after administration of distilled water orally or intraperitoneally. Significant ($P < 0.001$) reduction in systolic pressure, which was largely dissipated after four hours, followed intraperitoneal injection of 1 mg./Kg. of compound A-2275 (Table IV). Increase in dosage to 2 mg./Kg. intraperitoneally resulted in a slight increase in the extent and duration of the depressor response. Comparable effects were elicited by 4 and 8 mg./Kg. of compound A-2275 administered orally, indicating a moderate degree of gastrointestinal absorption. There was no significant difference in the degree or duration of blood pressure reduction produced by equivalent doses of piperoxan hydrochloride and compound A-2275.

Rat Hind Limb Perfusion.—The direct effect of compound A-2275 and piperoxan hydrochloride on peripheral blood vessels was determined by the use of a modification of the rat hind limb preparation described by Burn (6). In the present study, the abdominal aorta of the anesthetized rat was cannulated and the hind limbs perfused with oxygenated Ringer-Locke solution maintained at 37° and 60 mm. Hg pressure. The abdominal vein was cannulated with polyethylene tubing and arranged so that the perfusate collected in a cylindrical graduate. Volume of the perfusate was recorded at five-minute intervals. After the rate of flow became constant, the responsiveness of the preparation was tested by noting the transient decrease in flow rate following injection of 1–2 μg. of epinephrine hydrochloride into a side arm of the aortic cannula.

Piperoxan hydrochloride introduced into the system in amounts of 50, 100, and 250 μg. had no consistent effect on the rate of perfusion through the

[3] The authors wish to acknowledge the assistance of Dr. Sidney Goldstein during this phase of the investigation.

rat hind limb preparation (N=6). Relatively large doses, 0.5 and 1 mg., produced a slight reduction (0 to 8 and 5 to 17%, respectively) in flow rate. In contrast, compound A-2275 exhibited moderate direct vasoconstrictor activity. The amounts of compound A-2275 injected and the resulting decreases in perfusion rate were as follows: 50 μg.—10 to 12%; 100 μg.—16 to 26%; 250 μg.—32 to 45%; and 0.5 mg.—60 to 100% (N=8).

Direct stimulation of many different smooth muscles is a property common to many benzodioxans. Although the spasmogenic effect of compound A-2275 on vascular muscle would appear to interfere with its hypotensive activity, it is doubtful whether the blood concentrations achieved with effective depressor doses in the intact animal approximate those which were found to cause significant reduction of perfusion rate in the rat hind limb preparation.

SUMMARY

1. The compound 2-(N,N-diallylcarbamylmethyl) aminomethyl-1,4-benzodioxan (A-2275) was selected for pharmacologic evaluation from a series of new benzodioxan derivatives on the basis of hypotensive activity.

2. Oral, intraperitoneal, and intravenous LD_{50} values for compound A-2275 and piperoxan hydrochloride were determined in mice.

3. Compound A-2275, piperoxan hydrochloride, and tolazoline hydrochloride were compared in regard to their ability to provide protection against the lethal effect of epinephrine in mice.

4. Intravenous injection or oral administration of compound A-2275 produced moderate, but rather transient reduction of blood pressure, and partial inhibition of the pressor responses to epinephrine, norepinephrine, and carotid sinus occlusion in anesthetized normotensive dogs. Piperoxan hydrochloride, injected intravenously in subhypotensive doses, completely abolished the pressor responses to epinephrine and norepinephrine.

5. Comparable blood pressure reduction was obtained with equal doses of compound A-2275 and piperoxan hydrochloride administered orally and intraperitoneally to unanesthetized chronic hypertensive rats.

6. Compound A-2275 was found to possess moderate direct vasoconstrictor activity in the rat hind limb perfusion preparation. Piperoxan hydrochloride exhibited measurable spasmogenic activity only in relatively large concentrations.

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The Preparation of Oxidized Derivatives of 1-Ethylsulfonyl-4-ethylpiperazine*

By WILLIAM O. FOYE and LEO R. FEDOR, Jr.†

N-Substituted derivatives of 1-ethylsulfonylpiperazine have been prepared following the announcement of 1-ethylsulfonyl-4-ethylpiperazine as an antishock agent. Derivatives representing possible oxidation products of the N′-ethyl group were prepared by introducing carboxymethyl and hydroxyethyl groups into 1-ethylsulfonylpiperazine. The latter compound was obtained from 1-benzyloxycarbonylpiperazine, and the benzyloxycarbonyl group was removed by catalytic hydrogenation and by nonhydrolytic cleavage without cleavage of the ethylsulfonyl group.

THE EFFECTIVENESS of 1-ethylsulfonyl-4-ethylpiperazine in treating hemorrhagic shock in dogs was reported by Bovet, *et al.* (1), in 1948. Use of the same compound in the treatment of burn shock in rats was reported by Orth, *et al.* (2), in 1954. The latter group observed that the activity of this compound in preventing shock was delayed in warm weather when the metabolic rate of the rat is slower. This suggested that a metabolite was responsible for the antishock effects, and an oxidation product was considered a likely possibility. Attempts were made to prepare oxidized derivatives of the N-ethyl group, namely 1-ethylsulfonyl-4-β-hydroxyethylpiperazine and 1-ethylsulfonyl-4-piperazinoacetic acid.

Direct oxidation of the compound was considered to be impractical after attempts to oxidize 1-ethylpiperazine gave no characterizable

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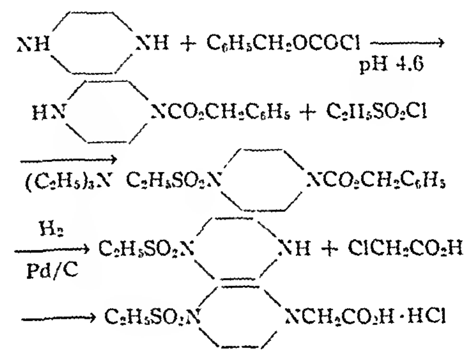
products. Substitution of an already oxidized side-chain on the free nitrogen of 1-ethylsulfonylpiperazine was then considered. The success of this procedure depends, of course, upon having a blocking group present on one of the nitrogens of the piperazine ring, prior to the introduction of the ethylsulfonyl group, which can be removed by a nonhydrolytic procedure. The blocking group most frequently employed for making monosubstituted or unsymmetrical N,N′-disubstituted piperazines has been the carbethoxyl, first introduced by Moore (3) in 1929. Few other easily removable groups have been found which may be monosubstituted in the piperazine ring in suitable yield. Disubstitution is, of course, the rule with piperazine reactions.

The use of 30 per cent hydrogen bromide in glacial acetic acid to cleave the carbethoxyl group from 1-ethylsulfonyl-4-carbethoxypiperazine had been found (4) to give rather poor yields of 1-ethylsulfonylpiperazine, and later attempts gave no better results. The alternative method of synthesis in which the oxidized side-chain is substituted into the piperazine ring first would be complicated by reaction of ethylsulfonyl chloride with the β-hydroxy group or salt of the carboxyl group. Protection of the hydroxyl group would later require a hydrolysis, probably causing elimination of the ethylsulfonyl group as well.

Use of the benzyloxycarbonyl group as a blocking agent was therefore investigated, since it may be cleaved from the piperazine nitrogen by catalytic hydrogenation. 1-Benzyloxycarbonylpiperazine was prepared by a method based on that of Goldman (5), which in turn was based upon the monocarbethoxylation procedure of Moore (3). A fair yield of the monosubstituted piperazine was obtained by conducting the reaction with benzyloxycarbonyl chloride at a controlled pH and separating the disubstituted derivative by extraction with chloroform. 1-Ethylsulfonyl-4-benzyloxycarbonylpiperazine was readily prepared, and both nonhydrolytic cleavage and catalytic hydrogenation procedures were used to remove the benzyloxycarbonyl group. The first-named procedure, using 30 per cent hydrogen bromide in glacial acetic acid, gave a 72 per cent conversion to 1-ethylsulfonylpiperazine hydrobromide, while low pressure hydrogenation over 10 per cent palladium-on-charcoal catalyst gave a 98.5 per cent yield. During the course of this work, Goldman and Williams (6) reported that a series of 1-carbamoyl-4-benzyloxycarbonylpiperazines could also be successfully cleaved by hydrogen and 10 per cent palladium-on-charcoal in refluxing aqueous solution.

The desired 1-ethylsulfonyl-4-β-hydroxyethylpiperazine was obtained by refluxing the 1-ethylsulfonylpiperazine with ethylene chlorohydrin, and 1-ethylsulfonyl-4-piperazinoacetic acid was obtained by heating 1-ethylsulfonylpiperazine with either chloro- or bromoacetic acid. The bromo acid gave a 45.5 per cent yield, while the chloro acid gave 80.5 per cent. Equation 1 illustrates the synthesis of 1-ethylsulfonyl-4-piperazinoacetic acid.



(Eq. 1)

It was also decided to prepare 1-β-hydroxyethylpiperazine and 1-piperazinoacetic acid for comparative purposes, and also because no biological testing has been reported for these compounds. The first named compound was prepared as the dihydrochloride according to the procedure of Moore (3), using 1-carbethoxypiperazine, ethylene chlorohydrin, and sodium carbonate, followed by aqueous hydrolysis of the carbethoxyl group. 1-Piperazinoacetic acid was obtained as the dihydrobromide from 1-carbethoxypiperazine and bromoacetic acid, followed by hydrogen bromide cleavage. Moore (3) obtained this compound by a much more involved procedure from 1-carbethoxypiperazine requiring a lengthy acid hydrolysis, neutralization with silver carbonate, and removal of excess silver.

Antishock tests have not yet been carried out with these compounds. However, four of the compounds, 1-piperazinoacetic acid dihydrobromide, 1-β-hydroxyethylpiperazine dihydrochloride, 1-ethylsulfonyl-4-piperazinoacetic acid hydrochloride, and 1-ethylsulfonyl-4-β-hydroxyethylpiperazine hydrochloride were tested against several parasitic infections in mice at the Lilly Research Laboratories. All of the compounds were ineffective against *T. equiperdum*, *S. novyi*, *S. obvelata*, and *A. tetraptera*, but 1-ethylsulfonyl-4-piperazinoacetic acid hydrochloride was effective against *E. histolytica* at a concentration of 62.5 µg./ml. *in vitro*.

EXPERIMENTAL

Melting points were taken on a Fisher-Johns block and are uncorrected Analyses were obtained from the Weiler and Strauss Microanalytical Laboratory, Oxford, England

1-Benzyloxycarbonylpiperazine.—A solution of 30 Gm (0 155 mole) of piperazine hexahydrate, 225 ml of methanol, 30 ml of water, and 46 5 ml of 6 *N* hydrochloric acid was heated to boiling, and 29 3 Gm (0 172 mole) of benzyloxycarbonyl chloride was added over a one half hour period along with sufficient 4 *N* sodium hydroxide to maintain the pH near 4 6 The mixture was heated for an additional three hours, allowed to stand overnight, and the methanol was then removed *in vacuo* The residue was made alkaline with sodium hydroxide, sodium carbonate was added, and the mixture was extracted with chloroform The chloroform extract was treated with dilute hydrochloric acid and washed with water Evaporation of the chloroform yielded 5 5 Gm of 1,4 bis (benzyloxycarbonyl) piperazine, m p 112–113° The acid extract was made alkaline with sodium hydroxide and extracted with chloroform The chloroform was evaporated under reduced pressure, and the oily residue was distilled The yield was 14 Gm (41%) of colorless product which boiled at 160–170° (2–3 mm), the recorded boiling point (5) is 158–161° (1 4 mm)

1-Ethylsulfonyl-4-benzyloxycarbonylpiperazine.—A solution of 13 95 Gm (0 063 mole) of 1 benzyloxycarbonylpiperazine, 100 ml of anhydrous ether, and 6 5 Gm (0 064 mole) of triethylamine was treated with an ether solution of 8 2 Gm (0 061 mole) of ethylsulfonyl chloride drop by drop The reaction temperature was kept at 15–20° by water cooling After the addition was complete, the reaction mixture was stirred for two hours at room temperature A precipitate of triethylamine hydrochloride was filtered from the solution, and white platelets then crystallized from the filtrate after partial evaporation Soxhlet extraction of the triethylamine hydrochloride with ether yielded an additional quantity of this material A total of 15 2 Gm (76 8%) of 1-ethylsulfonyl 4-benzyloxycarbonylpiperazine was obtained, m p 108–110°

Anal —Calcd for $C_{14}H_{20}N_2O_4S$ C, 53 85, H, 6 41 Found C, 53 87, H, 6 36

1-Ethylsulfonylpiperazine Hydrobromide by Nonhydrolytic Cleavage.—A solution of 1 ethylsulfonyl 4 benzyloxycarbonylpiperazine (3 4 Gm , 0 011 mole) glacial acetic acid (25 ml), and 30% hydrogen bromide in glacial acetic acid (5 ml) (Eastman Organic Chemicals) was allowed to stand at room temperature for fifteen minutes and in the refrigerator overnight The reaction was then warmed to room temperature, ether was added, and the precipitate was collected Recrystallization from ethanol yielded 2 Gm (72%) of 1 ethylsulfonylpiperazine hydrobromide, m p 218–220° The recorded m p (4) is 216–217 5°

1-Ethylsulfonylpiperazine by Catalytic Hydrogenation.—In a pressure bottle was placed 9 6 Gm (0 031 mole) of 1 ethylsulfonyl 4 benzyloxycarbonyl piperazine in 100 ml of ethanol The mixture was warmed to 45°, 2 Gm of 10% palladium on charcoal was added, and the mixture was hydrogenated for one half hour at three atmospheres pressure The catalyst was then removed by filtration, and the solvent was evaporated under reduced pressure A yield of 5 4 Gm (98 5%) of 1-ethylsulfonylpiperazine was obtained which boiled at 145–150° (2 mm) The hydrobromide melted at 217–219°, which agrees with the reported value (4)

1-Ethylsulfonyl-4-β-hydroxyethylpiperazine Hydrochloride.—A solution containing 1 2 Gm (0 007 mole) of 1-ethylsulfonylpiperazine and 5 Gm (0 062 mole) of ethylene chlorohydrin was refluxed for five hours The excess ethylene chlorohydrin was evaporated, and the resulting oil was crystallized from ethanol A yield of 0 5 Gm (29%) of crystalline solid was obtained which melted at 167–168°

Anal —Calcd for $C_8H_{19}N_2O_3SCl$ C, 37 33, H, 7 35 Found C, 37 38, H, 7 41

1-Ethylsulfonyl-4-piperazinoacetic Acid Hydrobromide.—A mixture of 1-ethylsulfonylpiperazine (0 5 Gm , 0 003 mole) and bromoacetic acid (0 4 Gm , 0 003 mole) was heated on a steam bath for fifteen minutes An oil was obtained which was crystallized with difficulty from ethanol, m p 176–177°, with previous softening The yield was 0 4 Gm or 45 5%

Anal —Calcd for $C_8H_{17}N_2O_4SBr$ C, 30 28, H, 5 36 Found C, 29 98, H, 5 77

1-Ethylsulfonyl-4-piperazinoacetic Acid Hydrochloride.—A solution of 1 ethylsulfonylpiperazine (0 5 Gm , 0 003 mole) and chloroacetic acid (0 26 Gm , 0 003 mole) in 25 ml of chloroform was refluxed for one hour The chloroform was evaporated, and the residue was crystallized from ethanol The yield was 0 62 Gm (80 5%) of product which melted at 132–133°

Anal —Calcd for $C_8H_{17}N_2O_4SCl$ C, 35 20, H, 6 24 Found C, 35 28, H, 6 14

1-Carbethoxy-4-β-hydroxyethylpiperazine —This compound was prepared by the procedure of Moore, Boyle, and Thorn (3) A 66% yield of colorless liquid was obtained b p 190–192° (20 mm) (lit (3) b p 184° (17 mm))

1-β-Hydroxyethylpiperazine Dihydrochloride —This product was obtained by the procedure of Moore, Boyle, and Thorn (3) A granular white solid was obtained which melted at 192–193° with previous softening No melting point has been reported for this compound

Anal —Calcd for $C_6H_{16}N_2OCl$ Cl, 34 9% Found Cl, 33 9%

1-Piperazinoacetic Acid Dihydrobromide —A mixture of 7 Gm (0 044 mole) of 1 carbethoxypiperazine (3) and 6 2 Gm (0 044 mole) of bromoacetic acid was heated on a steam bath for one half hour A glassy semisolid was obtained which was treated with 5 ml of 30% hydrogen bromide in glacial acetic acid The solution was heated at 75° for one half hour, and after cooling, 3 5 Gm (30%) of white solid was obtained which did not melt under 300°

Anal —Calcd for $C_6H_{14}N_2O\,Br_2$ N, 9 15 Found N, 9 18

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Tritium-Labeled Meprobamate*

By L. J. ROTH†, K. E. WILZBACH‡, A. HELLER†, and L. KAPLAN‡

Meprobamate has been labeled with tritium by exposure to tritium gas. Its radiochemical purity has been convincingly demonstrated, and the distribution of tritium within the molecule has been determined. The specific activity of 300 mc. per Gm. is the highest thus far reported for a product labeled by this technique.

THE SYNTHESIS of 2-methyl-2-*n*-propyl-1,3-propanediol dicarbamate (meprobamate) was described by Ludwig and Piech (1) The pharmacological properties of this tranquilizing drug have been described by Berger (2). Studies of its transport, metabolism, and cellular localization in the central nervous system are important and can be facilitated by use of a radioactive labeled compound. Because of the advantages of resolution which obtain with tritium in autoradiography, it was desirable to prepare radiochemically pure tritium-labeled meprobamate of high specific activity.

Labeled meprobamate with a specific activity of 50 μc. per Gm. has been prepared by the recoil triton method (3). Significantly higher activities are precluded in this method by the concomitant decomposition which occurs. Agranoff, Bradley, and Axelrod (4) have reported the labeling of meprobamate by exposure to tritium gas as described by Wilzbach (5), but do not mention the specific activity attained. We have now made a more detailed study of the labeling of meprobamate by this method in order to establish unequivocally the radiochemical purity of the product and to obtain information, needed for the interpretation of metabolic studies, of the distribution of the isotope within the molecule

PROCEDURES

Tritium Assay.—Tritium was determined by ion current measurement (6) on gas samples prepared by reductive decomposition of organic compounds with zinc and nickel oxide (7)

Paper chromatograms were scanned for tritium using the apparatus described by Kisieleski and Smetana (8)

Tritiation.—Meprobamate, obtained from Wallace Laboratories, New Brunswick, N J, was recrystallized from water (m p 105°, literature 105–106°) (1) One gram of the compound was exposed for seventy-two hours to 5.5 curies of tritium gas (4 17 cc S T P, 26 4% He, 8 5% H, 65 1% T) The tritium gas was removed and the product was treated twice with 50-ml portions of methanol to remove labile tritium Sixty millicuries of tritium was found in the methanol, the nonvolatile residue contained 350 mc

Purification.—The crude meprobamate was recrystallized once from water, then dissolved in methanol and treated with Norite, and subsequently subjected to a series of recrystallizations from water and benzene The specific activity of the product after each recrystallization is shown in Table I It is seen that after treatment with decolorizing carbon the product is essentially radiochemically pure The recovery of material at this point was 88%

Paper chromatography of the purified meprobamate with toluene-*n*-butanol-H_2O (19.1:20, upper phase) showed only a single radioactive peak with an R_f value of 0 35 (Fig. 1) Biological stability of the label was established by i.p. injection in a rat Unmetabolized meprobamate recovered from the urine by recrystallization had the same specific activity ($\pm 2\%$) as the injected dose.

TABLE I—PURIFICATION OF MEPROBAMATE-T BY SUCCESSIVE RECRYSTALLIZATION

Solvent	Activity, μc./mg
Water	344
Methanol + Norite	[a]
Water	305
Water	303
Benzene	304
Benzene	302
Water	303

[a] Not determined

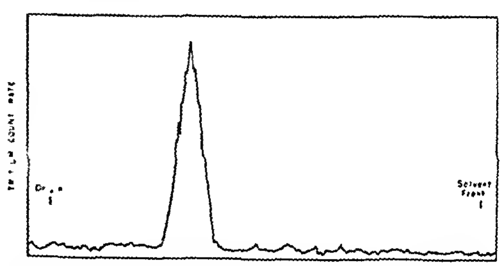

Fig 1—Radiometric scan of paper chromatogram of meprobamate T Solvent: toluene, *n*-butanol, water (19 1 20)

Preparation of Derivatives.—For the preparation of derivatives, 1 15 mg of the purified meprobamate-T was diluted with 2 913 Gm of the inactive compound and recrystallized from water and benzene The resultant product had a specific activity of 0 122 μc./mg (26 6 μc./mole) The following derivatives were prepared and assayed (Table II):

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† University of Chicago

‡ Argonne National Laboratory.

The authors are indebted to Mrs L F Roth for the tritium analyses and Mr H Lal for the biological isolation

TABLE II —DERIVATIVES OF MEPROBAMATE-T

Compound	Activity μc /mg	Activity μc /mole
Meprobamate	0 122	26 6
Diacetyl-1	0 089	26 9
Dixanthyl-2	0 0461	26 7
Diol 3	0 200	26 4
Diacid		
Acid Oxidation-4	0 109	17 5
Alkaline Oxidation-5	0 111	17 8

1 **N,N'-Diacetyl-2-methyl-2-n-propyl-1,3-propanediol Dicarbamate.**—This was prepared by acetylation with acetic anhydride and sulfuric acid and recrystallized from absolute alcohol, m p 134 135° (9)

2 **N,N'-Dixanthyl-2-methyl-2-n-propyl-1,3-propanediol Dicarbamate.**—This was prepared by heating on a steam bath for thirty minutes a solution of meprobamate and twice the calculated quantity of xanthydrol in a 1 1 mixture of glacial acetic acid and absolute ethanol Recrystallization from 95% alcohol gave a product, m p 182° (9)

3 **2-Methyl-2-n-propyl-1,3-propanediol.**—Meprobamate was hydrolyzed with alcoholic KOH The product was extracted with ether and recrystallized from hexane, m p 56°, Lit 62–63° (10)

4 **Methyl-n-propylmalonic Acid**—*Acid Oxidation* Cold fuming nitric acid (sp gr 1 50) was cautiously added to 300 mg of the 2-methyl-2-*n*-propyl propanediol in a small flask chilled in an ice bath until the diol was completely dissolved The flask was stoppered loosely and allowed to stand for two days at room temperature The mixture was made alkaline with KOH and washed twice with ether The solution was then acidified to pH 2 and extracted twice with ether The extracts were washed with water and dried over sodium sulfate The methyl-*n*-propylmalonic acid, obtained by evaporation of the extract, was recrystallized from benzene, m p 106°

5 **Methyl-n-propylmalonic Acid**—*Alkaline Oxidation* —To 300 mg of the diol dissolved in 15 ml of water were added 1 0 Gm of potassium permanganate in 70 ml of water and 0 9 Gm of anhydrous sodium carbonate This mixture was shaken thoroughly, stoppered, and allowed to stand at room temperature for one week The excess permanganate was destroyed by addition of 0 1*N* sodium bisulfite, the mixture was filtered, and the product was isolated and purified as above, m p. 106°

DISCUSSION

The recrystallization to constant specific activity (Table I), the identity of the molar activities of the derivatives with that of the meprobamate (Table II), and the presence of a single radiochemical peak in paper chromatography (Fig. 1) are convincing evidence for the radiochemical purity of the product The specific activity of 303 mc per Gm (one atom of tritium per 440 molecules of meprobamate) is the highest yet reported for this method This value corresponds to an incorporation rate of 18 mc of tritium per curie-day exposure, or a G value of 2 1 atoms of tritium per 100 electron volts The appearance of 74% of the total tritium incorporated from the gas, and 86% of the stably-bound tritium in meprobamate is unusually high for this method The material obtained is of sufficiently high specific activity for use in autoradiography, and results of such studies will be published at a later date The ease of purification and the high recovery of radiochemically pure material indicate that substantially higher specific activities could be attained if desired

Upon oxidation of the diol to the corresponding malonic acid, the loss of tritium from the hydroxymethylene groups is 33 8% (Table II) The ratio of tritium to hydrogen in these methylene groups is thus 1 28 times the average value for the alkyl substituents The fact that tritium is rather randomly distributed in the molecule should make it possible to detect any of the products resulting from metabolic changes in the diol moiety of meprobamate, since the label was also demonstrated to be biologically stable

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A Chromatographic Examination of the Alkaloidal Fraction of *Amanita pantherina**

By L. R. BRADY and V. E. TYLER, Jr.

A 1.5-Kg. quantity of oven-dried *Amanita pantherina* was defatted and extracted exhaustively with 70% ethanol. Purification of this extract yielded a solution which contained the total chloroform-soluble alkaloids of the mushroom. Chromatographic examination of this solution revealed the presence of two compounds giving typical alkaloid reactions and a third compound which appeared to be an indole derivative. Comparison of R_f values and color reactions of these compounds with known alkaloids revealed that neither bufotenine nor hyoscyamine, nor any alkaloid giving a positive Vitali-Morin reaction, was present in the extract.

THE PANTHER fungus, *Amanita pantherina* (Fr.) Quélet, grows abundantly in western Washington where it is responsible for a number of cases of mushroom poisoning annually. Collected in the button stage before the universal veil ruptures, the stipe lengthens and the pileus expands, it is commonly confused with puffballs and even truffles by the layman. Persons who have ingested it exhibit symptoms which are not entirely typical of muscarine poisoning in that they experience vivid hallucinations of the type induced by certain tropane (hyoscyamine, scopolamine) or indole (bufotenine, N,N-dimethyltryptamine) alkaloids (1).

Both of these types of alkaloids have been reported to occur in *A. pantherina*. In 1953, Wieland and Motzel (2) claimed to have detected, by chromatographic means, the presence of bufotenine in *A. pantherina* as well as in *Amanita muscaria* (Fr.) S. F. Gray. These investigators did not publish experimental details of this work, and the presence of the compound has not been confirmed by others.

The literature pertaining to pilzatropine, the hypothetical alkaloid of *A. pantherina* and *A. muscaria*, has been reviewed recently (3). Lewis (4) reported in 1955 the isolation of *l*-hyoscyamine from small samples of these two species, apparently confirming the claims of some previous investigators who reported the separation of alkaloids with atropine-like activity from the muscarine fraction of these mushrooms. However, a year prior to Lewis's report, Kwasniewski (5) had been unable to establish the presence of a base with mydriatic activity in much larger quantities of *A. muscaria*.

Due to the difficulties involved in collecting a sufficient quantity of the mushrooms to allow the application of classical analytical procedures, it was decided to employ existing paper chromatographic methods suitable for the identification of extremely small quantities of the tropane and indole alkaloids.

EXPERIMENTAL

Preparation of the Alkaloidal Extract.—A quantity of *A. pantherina* was collected during the winter and spring of 1957–1958 in the vicinity of Olympia, Washington. Several of the collections were made in areas where people had previously obtained panther fungus which, when eaten, produced typical hallucinations. The individual specimens were carefully cleaned, sliced, and dried in a forced-air drying oven at 48° for three to five days. One hundred and sixty-five pounds of fresh mushrooms yielded seven pounds of oven-dried material.

The dried fungus was ground to a coarse powder in a Wiley mill, and a 1.5-Kg. quantity was moistened with petroleum ether and packed in a large percolator. Following a maceration period, the drug was extracted with petroleum ether which was allowed to percolate at a moderate rate until 7.35 L. were collected.

After defatting, the marc was removed from the percolator and allowed to air dry. It was then moistened with 70% ethanol, repacked in the percolator, allowed to macerate for twenty hours, and extracted at a moderate rate with 70% ethanol until 14 L. were collected. This ethanolic extract was concentrated to a thick syrupy liquid at 60° under reduced pressure in a Flash-Evaporator.

The residue was taken up in 2 L. of 1% hydrochloric acid, insoluble resinous matter removed by filtration, and the acid filtrate was shaken out with four successive 200-ml. portions of chloroform to remove traces of fats and other nonalkaloidal ballast materials. After separation of the last portion of chloroform, the aqueous solution was rendered distinctly alkaline with ammonium hydroxide and extracted with five successive 200-ml. portions of chloroform. The combined chloroform extracts were evaporated to a 10-ml. volume at 45° under reduced pressure in a Flash-Evaporator.

One half (5 ml.) of this crude alkaloidal extract was purified further by placing it in a separatory funnel and extracting with three successive portions (5, 3, and 2 ml.) of 2% hydrochloric acid. The

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combined acid extract was then made alkaline with ammonium hydroxide and extracted with three successive portions (2, 2, and 1 ml) of chloroform The combined solution which would contain any chloroform-soluble alkaloids originally present in the mushrooms, including the bufotenine and *l*-hyoscyamine reported by previous investigators, was subjected to chromatographic analysis Each ml represented 150 Gm of oven dried plant material or approximately 3 5 Kg of fresh material

Chromatographic Procedures.—Replicate 100 μl quantities of the alkaloidal extract prepared as described above, (equivalent to 350 Gm fresh plant material) together with known samples of hyoscyamine, scopolamine, and bufotenine were spotted in successive 10 μl portions on sheets of Whatman No 1 filter paper The reference alkaloids were applied to the sheets singly and as mixtures with themselves and with the extract of the plant material No appreciable differences in the R_f values of the alkaloids were observed under these different conditions The sheets were then subjected to ascending formation with a wash liquid composed of the upper phase of a mixture of *n* butanol glacial acetic acid water (4 1 5) After a period of approximately eighteen to twenty hours, they were removed from the chromatographic chamber, dried, and treated with various reagents These included (*a*) Thies and Reuther's modification of the Dragendorff reagent (6) a general reagent forming orange colored spots with most alkaloids, (*b*) 1% cinnamic aldehyde in methanol followed by exposure to hydrochloric acid vapor (7), giving orange to pink spots with many indole derivatives (*c*) 2% *p* dimethylamino benzaldehyde in 1 2 N hydrochloric acid (8), which forms blue to purple colored spots with many indole derivatives (*d*) Pauly s reagent (9), which reacts to form orange or pinkish spots with compounds containing a phenolic hydroxy group or with imidazoles

After spraying with these reagents, three distinct spots were observed on the chromatogram of the purified alkaloidal extract of *A pantherina*, but none of them corresponded exactly in R_f values or in color reactions to hyoscyamine, scopolamine, or bufotenine See Table I

Two spots, apparently alkaloidal in character, were observed which exhibited R_f values in the general region of scopolamine and hyoscyamine It was decided to test these spots to determine if they represented alkaloids of the solanaceous type This was carried out by spotting 150 μl quantities of the purified alkaloidal extract on paper and chromatographing as described The areas representing the two alkaloidal spots were cut from the paper and eluted with ethanol After evaporation of the ethanol, the Vitali-Morin test was carried out on the residues as described by Cromwell (10)

Two additional reagents were employed in an attempt to learn something about the nature of the compounds which gave the positive tests for alkaloids Strips were treated with a sodium nitroprusside acetaldehyde reagent (11) which gives positive reactions with secondary aliphatic and alicyclic amines and with Konig's reagent (12) which is useful for detecting pyridine derivatives

Since the R_f values of a number of indole derivatives are quite similar in the butanol acetic acid water system, it appeared worth while to examine the purified alkaloidal extract in a system better suited for the separation of these compounds A *n* propanol-1 *N* ammonia (4 1) system was consequently employed for the examination of the extract in the same manner described for the previous solvent system R_f values and color reactions obtained with this wash liquid are recorded in Table I

DISCUSSION

The extract of *A pantherina*, containing the total chloroform soluble alkaloids of that plant, was subjected to chromatographic examination in a butanol acetic acid water system Two spots were obtained which gave typical alkaloidal reactions with Thies and Reuther's reagent In addition, a third somewhat diffuse spot was present which gave reactions typical of the indole nucleus with *p* dimethylaminobenzaldehyde as well as a positive reaction with Pauly's reagent, possibly indicative of a phenolic hydroxy group No positive reactions were observed when the strips were treated with the sodium nitroprusside acetaldehyde or with the Konig s reagent, and it was concluded that the unknown compounds were not secondary amines or derivatives of pyridine

When subjected to analysis in a *n* propanol 1 N ammonia system, the compounds giving positive Thies and Reuther's reactions apparently failed to

TABLE I —COLOR REACTIONS AND R_f VALUES OF COMPOUNDS IN ALKALOID FRACTION OF *Amanita pantherina* AND OF REFERENCE COMPOUNDS

Identity of Material	Av R_f of Spot	Color Reaction with Reagent: Thies and Reuther's	Cinnamic Aldehyde + HCl	*p* Dimethyl aminobenz aldehyde	Pauly s
	n Butanol Acetic Acid-Water (4 1 5)				
Purified alkaloidal extract	0 87	none	purple	blue	pink
	0 68	orange	none	none	none
	0 51	orange	none	none	none
Hyoscyamine	0 64	orange	none	none	none
Scopolamine	0 55	orange	none	none	none
Bufotenine	0 41	orange	orange	purple	orange pink
	n-Propanol 1 *N* Ammonia (4 1)				
Purified alkaloidal extract	0 86	orange	none	none	none
	0 83	none	purple	blue	pink
Hyoscyamine	0 85	orange	none	none	none
Scopolamine	0 80	orange	none	none	none
Bufotenine	0 75	orange	orange	purple	orange-pink

separate since only one such spot was observed. The indole derivative again appeared as a single spot and still gave a positive Pauly's reaction.

The R_f values and color reactions of the spots in both chromatographic systems failed to correspond exactly with those observed for hyoscyamine and bufotenine, the alkaloids previously reported to exist in this mushroom. The spots which gave typical alkaloid reactions in the butanol-acetic acid-water system and which possessed R_f values quite similar to hyoscyamine and scopolamine, were eluted and subjected to the Vitali-Morin test for solanaceous alkaloids. In spite of the sensitivity of this reaction which will detect as little as 0.0001 mg. of hyoscyamine or scopolamine, the tests were completely negative, no purple color being observed.

Two unidentified compounds possessing general alkaloidal solubilities and giving typical alkaloidal reactions were shown to be present in trace quantities in the sample of *A. pantherina* investigated. A third compound, giving positive reactions for the indole nucleus as well as a positive Pauly's reaction was present in the alkaloid fraction. The latter constituent failed to give a typical alkaloidal reaction with Thies and Reuther's reagent, and the purple color which it exhibited with cinnamic aldehyde was not typical of simple indole derivatives. On the basis of these observations, it was concluded that neither bufotenine nor hyoscyamine, nor any alkaloid giving a positive Vitali-Morin reaction and which might be designated "pilzatropine," was present in the alkaloidal extract. These results are in disagreement with those of Lewis and Wieland and Motzel. Although it is possible that the differences may be due to the existence of strains of *A. pantherina* possessing different biosynthetic abilities, this remains to be determined by the analysis of mushrooms collected in different localities but subjected to uniform analytical procedures.

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Comparative Activities and Toxicities III*

D-Glucamine and Glycine Derivatives of Nitro and Halogenated Benzenes

By EDWARD G. FELDMANN and WILLIAM O. FOYE

D-Glucamine and glycine molecules have been introduced into several nitro and halogenated benzene derivatives in an attempt to reduce the toxic effects to the host of such compounds. A comparison of the acute toxicities of the two types of derivatives showed that the sugar group exerted a definite detoxifying effect, whereas the glycine and glycine ester groups had little effect on toxicity. The antibiotic activities of *m*-dinitrobenzene, 2,4-dinitroaniline, and *p*-nitroaniline were wholly removed when the D-glucamine group was present in these molecules, but N-(2-nitro-4,5-dichlorophenyl)-D-glucamine was found to have a relatively broad antibiotic spectrum. The *o*-, *m*-, and *p*-nitrophenylglycines and their esters revealed a rather limited activity against microorganisms.

MICROBIOLOGICAL INHIBITIONS by aromatic nitro compounds and halides have become a subject for serious investigation since the successful introduction of Chloromycetin and the nitrofurans as antibacterial agents. Examples of this type of compound that have been studied are nitrobenzoic acid derivatives (1), nitroquinolines (2), N-arylglycines (3), and a series of simple nitrobenzene derivatives including, in particular, *m*-dinitrobenzene (4). The metabolism of various halogenated nitrobenzenes has also been investigated (5). Since the toxicity of these aromatic nitro and halogenated compounds to the host is often considerable, an attempt has been made to lower the toxicity of simple aromatic nitro and halogenated compounds by the inclusion of sugar and amino acid residues in these molecules, and perhaps provide a more useful compound of this type. This report describes the results of this attempt. Previously, sugar and amino acid deriva-

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TABLE I.—ACUTE INTRAPERITONEAL TOXICITIES OF NITRO AND HALOGENATED BENZENE DERIVATIVES[a]

No	Compound	Dose in Mice, mg./Kg.	No. Died[b]/ No. Used	Toxic Signs
1.	*m*-Dinitrobenzene	200	1/5	Death in 2 hr.
		400	4/5	
2.	2,4-Dinitroaniline	200	0/5	Death in 24 hr.
		400	5/5	
3.	N-(2,4-Dinitrophenyl)-D-glucamine	800	0/5	....
4.	*p*-Nitroaniline	400	0/5	Death in 2 hr.
		800	5/5	
5.	N-(4-Nitrophenyl)-D-glucamine	800	0/5	None
6.	*p*-Bromobenzamide	400	2/5	Deaths 24–48 hr.
		800	3/5	Deaths in 4 hr.
7.	N-(4-Bromobenzoyl)-D-glucamine	800	0/5	Lethargy and ataxia
8.	N-(2-Nitro-4,5-dichlorophenyl)-D-glucamine	400	0/5	Deaths delayed 24 hr.
		800	5/5	

[a] Determined at the Lilly Research Laboratories by R C Anderson
[b] Observations were made for one week after single injections of compound

tives of amino- and nitrosalicylic acids (6) and bis-(4-aminophenyl)-sulfone (7) were prepared for the same purpose.

The detoxifying effect of a sugar residue may be seen in Table I, where the acute i.p. toxicities in mice are shown for a series of nitro and halogenated benzenes and their D-glucamine derivatives. A consistent lowering of toxicity was observed when the glucose residue was included in the *p*-nitroaniline, 2,4-dinitroaniline, and *p*-bromobenzamide molecules. Although a limited number of examples is presented, the principle of detoxication by introduction of a sugar molecule into a toxic compound seems to be satisfactorily illustrated. Previous studies, already referred to (6, 7), also showed a consistent lowering of acute toxicity by introduction of sugars into fairly toxic molecules.

The detoxifying effect of an amino acid residue on the nitroaniline molecule was observed after preparation of a series of N-nitrophenyl glycines and their ethyl esters. As may be seen in Table II, no appreciable detoxifying effect on the *p*-nitroaniline molecule was exerted by inclusion of either the glycine or glycine ester residue. Although i.p. toxicities were not determined for *o*- and *m*-nitroaniline, it is unlikely that they would differ a great deal from that of *p*-nitroaniline.

The results of antimicrobial screening (agar dilution technique) of the nitrobenzenes and their D-glucamine derivatives are shown in Table III. A definite narrowing of the antibiotic spectrum is evident when *m*-dinitrobenzene is converted to 2,4-dinitroaniline, and all activity disappeared on further conversion to N-(2,4-dinitrophenyl)-D-glucamine, when testing levels up to 200 μg./ml. were used. Similarly, no activity was shown by N-(4-nitrophenyl)-D-glucamine, and *p*-bromobenzamide and its D-glucamine derivative were also relatively inactive. However, N-(2-nitro-4,5-dichlorophenyl)-D-glucamine was found to have a relatively good antibiotic spectrum, and it showed an appreciable activity against mycobacteria. This activity did not remain when the compound was tested against tuberculosis in mice, unfortunately. The acute i.p. toxicity of this derivative was not much greater than that of the other nitrophenyl-D-glucamine derivatives tested. Antiviral tests were also carried out with the 4-nitrophenyl, 2,4-dinitrophenyl, 4-bromobenzoyl, and 2-nitro-4,5-dichlorophenyl D-glucamines, and all four compounds were ineffective against two types of virus in mice.

TABLE II.—ACUTE INTRAPERITONEAL TOXICITIES OF NITROPHENYL GLYCINES[a]

No.	Compound	$LD_{50} \pm$ S. E (in Mice), mg./Kg.
9.	N-(*o*-Nitrophenyl)-glycine	469.9 ± 45.1
10.	N-(*m*-Nitrophenyl)-glycine	739.1 ± 226.1
11.	N-(*p*-Nitrophenyl)-glycine	631.7 ± 106.5
12.	Ethyl N-(*o*-nitrophenyl)-glycinate	500.0 ± 57.0
13.	Ethyl N-(*m*-nitrophenyl)-glycinate	524.8 ± 84.3
14.	Ethyl N-(*p*-nitrophenyl)-glycinate	$632\ 4 \pm 66.4$

[a] Determined at the Lilly Research Laboratories by R. C. Anderson.

Antimicrobial screening of the N-(nitroaryl)-glycines and their esters revealed a rather limited activity against microorganisms. This is evident from Table IV, where only test organisms which were inhibited are listed. The esters in general showed a somewhat broader spectrum than the free acids, although in neither case did the breadth of activity approach that of the most successful D-glucamine derivative. It is interesting to note that N-(*m*-nitrophenyl)-glycine showed no activity whatsoever, and it has also been shown that the *m*-nitrophenyl halides are metabolized in a different manner than the *o*- and *p*-isomers (5). Some other sugar derivatives, prepared for a pre-

TABLE III.—ANTIBIOTIC SPECTRA OF NITRO AND HALOGENATED BENZENE DERIVATIVES[a]

Test Organism	Inhibitory Concentration µg./ml.[b] Compd. No. 1	2	6	8
Staphylococcus aureus				50
Staphylococcus albus				50
Bacillus subtilis	200	200		6.25
Mycobacterium phlei	200			0.2
Mycobacterium tuberculosis	200	200		0.2
Mycobacterium avium	200	200		3.13
Escherichia coli				200
Proteus vulgaris	200			100
Pseudomonas aeruginosa				200
Aerobacter aerogenes				>200
Klebsiella pneumoniae				200
Salmonella enteritidis				>200
Shigella paradysenteriae	50			25
Saccharomyces pastorianus	100			25
Candida albicans	50			100
Trichophyton rubrum	50	200		12.5
Trichophyton interdigitale	200	200		12.5
Brucella bronchiseptica		200		200
Vibrio metschnikovi		200		50
Erwinia amylovora	200	200		200
Agrobacterium tumefaciens	100	200		200
Xanthomonas campestris	200	200		200
Xanthomonas malvacearum	c	c		200
Xanthomonas phaseoli	c	c		200
Pseudomonas solanacearum	50	c		200
Pseudomonas syringae	>200			>200
Corynebacterium insidiosum	100			200
Corynebacterium sepodonicum	100	c		100
Aspergillus niger	10	200	200	50
Ustilago avenae	100	c		50
Alternaria solani	c	c		50
Ceratostomella fimbriata	100		200	50
Fusarium moniliforme	>200			50
Fusarium oxysporum lycopersici	200	200		200
Colletotrichum gossypii	10	c		50
Glomerella singulata	50			50
Verticillium albo-atrum	100	200		50
Sclerotium bataticola	c	c		c

[a] Carried out at the Lilly Research Laboratories by F. Kornann.
[b] The agar dilution technique was used, the bacteria being observed for forty-eight hours, and the bacterial and fungal plant pathogens for seventy-two hours.
[c] Test organism did not grow sufficiently well to establish an end point. Where no inhibitory concentration is reported the organism was not affected by 200 µg./ml.

vious investigation (6), namely 1,2,5,6-di-O-isopropylidene-3-(2-benzyloxy-4-nitrobenzoyl)-D-glucofuranose, monoacetone glucose, and diacetone glucose, were found to be devoid of activity in the antimicrobial screening test employed.

It may be concluded from these comparisons that the introduction of a glucose residue into a nitrobenzene derivative generally lowers the acute toxicity to a considerable extent. The relative position of the nitro group in a mononitro compound appears to have little effect on the toxicity. The antimicrobial activity of nitrobenzene derivatives was found to undergo considerable change

TABLE IV.—ANTIBIOTIC SPECTRA OF NITROPHENYL GLYCINES[a]

Test Organism	Inhibitory Concentration, µg./ml.[b] Compd. No. 9	11	12	13	14
Mycobacterium phlei			200		
Mycobacterium tuberculosis			200	200	
Mycobacterium avium			200	200	
Agrobacterium tumefaciens			200		
Xanthomonas malvacearum	200	200	200	200	
Xanthomonas phaseoli				200	
Corynebacterium insidiosum		200			200
Corynebacterium sepodonicum	200	200	200	200	200
Aspergillus niger			200		
Ustilago avenae	200	200	200	200	200
Alternaria oleracea	200	200	200	200	200

[a] Carried out at the Lilly Research Laboratories by Sue Burden.
[b] The same footnote applies here as for Table III.

with the introduction of a detoxifying group, and this activity is apparently a structurally specific effect and not a general one for nitro aromatics. The combination of nitro and halogen groups in the same molecule gave a superior antibiotic spectrum to that of molecules containing either nitro or halogen groups alone.

METHODS OF PREPARATION

The D-glucamine derivatives were obtained from the reaction of D-glucamine with nitroaryl halides, such as 2,4-dinitrochlorobenzene and 1,2-dichloro-4,5-dinitrobenzene. This reaction has been described previously (8), and was found to give primary aromatic amines more often than D-glucamine derivatives, but it was possible to obtain the N-4-nitrophenyl, N-2,4-dinitrophenyl, and N-2-nitro-4,5-dichlorophenyl D-glucamines by this method using either pyridine or ethanol-sodium acetate as solvent.

Preparation of the nitrophenylglycines was first attempted with the reaction of the respective nitrophenyl chlorides with glycine in the presence of pyridine. The reaction mixture, after refluxing, showed considerable decomposition, and pure products were not isolated.

Reaction of nitroanilines with chloroacetic acid in aqueous sodium acetate solution, however, gave low yields of products which were increased by varying reaction conditions. Isolation and purification of the crude products was achieved by conversion to the water-soluble sodium salts and reconversion to the insoluble acids, followed by recrystallization. The nitrophenylglycines were easily converted to ethyl esters.

EXPERIMENTAL

The melting points were taken on a Fisher-Johns block which gave correct values for a set of U. S. P. melting point standards. Analyses were carried out at the Weiler and Strauss Microanalytical Laboratory, Oxford, England.

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		400	5/5	
3.	N-(2,4-Dinitrophenyl)-D-glucamine	800	0/5	....
4.	*p*-Nitroaniline	400	0/5	Death in 2 hr.
		800	5/5	
5.	N-(4-Nitrophenyl)-D-glucamine	800	0/5	None
6.	*p*-Bromobenzamide	400	2/5	Deaths 24–48 hr.
		800	3/5	Deaths in 4 hr.
7.	N-(4-Bromobenzoyl)-D-glucamine	800	0/5	Lethargy and ataxia
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[a] Determined at the Lilly Research Laboratories by R. C Anderson
[b] Observations were made for one week after single injections of compound

tives of amino- and nitrosalicylic acids (6) and bis-(4-aminophenyl)-sulfone (7) were prepared for the same purpose.

The detoxifying effect of a sugar residue may be seen in Table I, where the acute i.p. toxicities in mice are shown for a series of nitro and halogenated benzenes and their D-glucamine derivatives. A consistent lowering of toxicity was observed when the glucose residue was included in the *p*-nitroaniline, 2,4-dinitroaniline, and *p*-bromobenzamide molecules. Although a limited number of examples is presented, the principle of detoxication by introduction of a sugar molecule into a toxic compound seems to be satisfactorily illustrated. Previous studies, already referred to (6, 7), also showed a consistent lowering of acute toxicity by introduction of sugars into fairly toxic molecules.

The detoxifying effect of an amino acid residue on the nitroaniline molecule was observed after preparation of a series of N-nitrophenyl glycines and their ethyl esters. As may be seen in Table II, no appreciable detoxifying effect on the *p*-nitroaniline molecule was exerted by inclusion of either the glycine or glycine ester residue. Although i.p. toxicities were not determined for *o*- and *m*-nitroaniline, it is unlikely that they would differ a great deal from that of *p*-nitroaniline.

The results of antimicrobial screening (agar dilution technique) of the nitrobenzenes and their D-glucamine derivatives are shown in Table III. A definite narrowing of the antibiotic spectrum is evident when *m*-dinitrobenzene is converted to 2,4-dinitroaniline, and all activity disappeared on further conversion to N-(2,4-dinitrophenyl)-D-glucamine, when testing levels up to 200 μg./ml. were used. Similarly, no activity was shown by N-(4-nitrophenyl)-D-glucamine, and *p*-bromobenzamide and its D-glucamine derivative were also relatively inactive. However, N-(2-nitro-4,5-

TABLE II.—ACUTE INTRAPERITONEAL TOXICITIES OF NITROPHENYL GLYCINES[a]

No.	Compound	LD_{50} + S. E. (in Mice), mg./Kg
9.	N-(*o*-Nitrophenyl)-glycine	469.9 ± 45.1
10.	N-(*m*-Nitrophenyl)-glycine	739.1 ± 226.1
11.	N-(*p*-Nitrophenyl)-glycine	631.7 ± 106.5
12.	Ethyl N-(*o*-nitrophenyl)-glycinate	500.0 ± 57.0
13.	Ethyl N-(*m*-nitrophenyl)-glycinate	524.8 ± 84.3
14.	Ethyl N-(*p*-nitrophenyl)-glycinate	632.4 ± 66.4

[a] Determined at the Lilly Research Laboratories by R. C Anderson

dichlorophenyl)-D-glucamine was found to have a relatively good antibiotic spectrum, and it showed an appreciable activity against mycobacteria. This activity did not remain when the compound was tested against tuberculosis in mice, unfortunately. The acute i.p. toxicity of this derivative was not much greater than that of the other nitrophenyl-D-glucamine derivatives tested. Antiviral tests were also carried out with the 4-nitrophenyl, 2,4-dinitrophenyl, 4-bromobenzoyl, and 2-nitro-4,5-dichlorophenyl D-glucamines, and all four compounds were ineffective against two types of virus in mice.

Antimicrobial screening of the N-(nitroaryl)-glycines and their esters revealed a rather limited activity against microorganisms. This is evident from Table IV, where only test organisms which were inhibited are listed. The esters in general showed a somewhat broader spectrum than the free acids, although in neither case did the breadth of activity approach that of the most successful D-glucamine derivative. It is interesting to note that N-(*m*-nitrophenyl)-glycine showed no activity whatsoever, and it has also been shown that the *m*-nitrophenyl halides are metabolized in a different manner than the *o*- and *p*-isomers (5). Some other sugar derivatives, prepared for a pre-

TABLE III.—ANTIBIOTIC SPECTRA OF NITRO AND HALOGENATED BENZENE DERIVATIVES[a]

Test Organism	Inhibitory Concentration µg./ml.[b] Compd. No. 1	2	6	8
Staphylococcus aureus				50
Staphylococcus albus				50
Bacillus subtilis	200	200		6.25
Mycobacterium phlei	200			0.2
Mycobacterium tuberculosis	200	200		0.2
Mycobacterium avium	200	200		3.13
Escherichia coli				200
Proteus vulgaris	200			100
Pseudomonas aeruginosa				200
Aerobacter aerogenes				>200
Klebsiella pneumoniae				200
Salmonella enteritidis				>200
Shigella paradysenteriae	50			25
Saccharomyces pastorianus	100			25
Candida albicans	50			100
Trichophyton rubrum	50	200		12.5
Trichophyton interdigitale	200	200		12.5
Brucella bronchiseptica		200		200
Vibrio metschnikovii		200		50
Erwinia amylovora	200	200		200
Agrobacterium tumefaciens	100	200		200
Xanthomonas campestris	200	200		200
Xanthomonas malvacearum	c	c		200
Xanthomonas phaseoli	c	c		200
Pseudomonas solanacearum	50	c		200
Pseudomonas syringae	>200			>200
Corynebacterium insidiosum	100			200
Corynebacterium sepedonicum	100	c		100
Aspergillus niger	10	200	200	50
Ustilago avenae	100	c		50
Alternaria solani	c	c		50
Ceratostomella fimbriata	100		200	50
Fusarium moniliforme	>200			50
Fusarium oxysporum lycopersici	200	200		200
Colletotrichum gossypii	10	c		50
Glomerella singulata	50			50
Verticillium albo-atrum	100	200		50
Sclerotium bataticola	c	c		c

[a] Carried out at the Lilly Research Laboratories by F. Kornann.
[b] The agar dilution technique was used, the bacteria being observed for forty-eight hours, and the bacterial and fungal plant pathogens for seventy-two hours.
[c] Test organism did not grow sufficiently well to establish an end point. Where no inhibitory concentration is reported the organism was not affected by 200 µg./ml.

vious investigation (6), namely 1,2,5,6-di-O-isopropylidene-3-(2-benzyloxy-4-nitrobenzoyl)-D-glucofuranose, monoacetone glucose, and diacetone glucose, were found to be devoid of activity in the antimicrobial screening test employed.

It may be concluded from these comparisons that the introduction of a glucose residue into a nitrobenzene derivative generally lowers the acute toxicity to a considerable extent. The relative position of the nitro group in a mononitro compound appears to have little effect on the toxicity. The antimicrobial activity of nitrobenzene derivatives was found to undergo considerable change

TABLE IV.—ANTIBIOTIC SPECTRA OF NITROPHENYL GLYCINES[a]

Test Organism	Inhibitory Concentration, µg./ml.[b] Compd. No. 9	11	12	13	14
Mycobacterium phlei			200		
Mycobacterium tuberculosis			200	200	
Mycobacterium avium			200	200	
Agrobacterium tumefaciens			200		
Xanthomonas malvacearum	200	200	200	200	
Xanthomonas phaseoli				200	
Corynebacterium insidiosum		200			200
Corynebacterium sepedonicum	200	200	200	200	200
Aspergillus niger			200		
Ustilago avenae	200	200	200	200	200
Alternaria oleracea	200	200	200	200	200

[a] Carried out at the Lilly Research Laboratories by Sue Burden.
[b] The same footnote applies here as for Table III.

with the introduction of a detoxifying group, and this activity is apparently a structurally specific effect and not a general one for nitro aromatics. The combination of nitro and halogen groups in the same molecule gave a superior antibiotic spectrum to that of molecules containing either nitro or halogen groups alone.

METHODS OF PREPARATION

The D-glucamine derivatives were obtained from the reaction of D-glucamine with nitroaryl halides, such as 2,4-dinitrochlorobenzene and 1,2-dichloro-4,5-dinitrobenzene. This reaction has been described previously (8), and was found to give primary aromatic amines more often than D-glucamine derivatives, but it was possible to obtain the N-4-nitrophenyl, N-2,4-dinitrophenyl, and N-2-nitro-4,5-dichlorophenyl D-glucamines by this method using either pyridine or ethanol-sodium acetate as solvent.

Preparation of the nitrophenylglycines was first attempted with the reaction of the respective nitrophenyl chlorides with glycine in the presence of pyridine. The reaction mixture, after refluxing, showed considerable decomposition, and pure products were not isolated.

Reaction of nitroanilines with chloroacetic acid in aqueous sodium acetate solution, however, gave low yields of products which were increased by varying reaction conditions. Isolation and purification of the crude products was achieved by conversion to the water-soluble sodium salts and reconversion to the insoluble acids, followed by recrystallization. The nitrophenylglycines were easily converted to ethyl esters.

EXPERIMENTAL

The melting points were taken on a Fisher-Johns block which gave correct values for a set of U. S. P. melting point standards. Analyses were carried out at the Weiler and Strauss Microanalytical Laboratory, Oxford, England.

The preparation of D-glucamine, N-(4-nitrophenyl)-D-glucamine, and N-(2,4-dinitrophenyl)-D-glucamine have already been reported (8). The preparation of N-(2-nitro-4,5-dichlorophenyl)-D-glucamine was carried out according to the method of Holly, *et al.* (9), and a 52% yield of product melting at 188–191° was obtained (Holly reported a m. p. of 193–195°).

p-Bromobenzamide.—*p*-Bromobenzoic acid was prepared in 82% yield using the method of Chiang and Tseng (10), m. p. 246–250° (lit. (10) m. p. 251–253°). *p*-Bromobenzamide was prepared by conversion of *p*-bromobenzoic acid to the acyl chloride using a large excess of thionyl chloride according to the procedure of Meyer (11). This was then converted to the amide using concentrated ammonium hydroxide according to the method of Schotten (12). After recrystallization from aqueous ethanol, the overall yield from the acid was 47%, m. p. 186–188° (lit. (12) m. p. 189°).

N-(4-Bromobenzoyl)-D-glucamine.—D-Glucamine (10.0 Gm., 0.055 mole) and *p*-bromobenzoic acid (5.0 Gm., 0.025 mole) were placed in a flask with 15 ml. of anhydrous pyridine. The mixture was refluxed for eighteen hours, and the resulting solution was diluted with 15 ml. of water and steam distilled until 250 ml. of distillate was collected. The remaining solution was concentrated to about 20 ml. and chilled. A small quantity of precipitate was recrystallized from methanol to give 0.2 Gm. of white powder, m. p. 207–208°. This material gave a positive test for halogen and was not soluble in sodium hydroxide solution. Repeated recrystallization from methanol failed to raise the m. p. appreciably.

Anal.—Calcd. for $C_{13}H_{18}NO_6Br$: C, 42.87; H, 4.98. Found: C, 42.85; H, 5.02.

N-(o-Nitrophenyl)-glycine.—A mixture of *o*-nitroaniline (16.0 Gm., 0.116 mole), sodium acetate trihydrate (50.0 Gm., 0.368 mole), and water (200 ml.) was heated to boiling, and a solution of 32 Gm. (0.339 mole) of chloroacetic acid in 60 ml. of water was introduced, dropwise, over a period of two hours. Heating and stirring were continued for another hour, and the solution was adjusted to a pH of 9 using ammonium hydroxide solution. After cooling overnight, the reaction mixture was filtered, the filtrate acidified, and the precipitate collected. The residue first collected was extracted with 4% ammonia, and the extracts were acidified. The resulting precipitate was combined with the previous product from acidification, and the combined product was dissolved in aqueous ammonia, filtered, and reprecipitated with acid. The product was washed repeatedly with water, and further purified by reprecipitation and recrystallization from hot water to give 1.65 Gm. (7%) of deep orange platelets, m. p. 196–199° (lit. (13) m. p. 192–193°).

Ethyl N-(o-Nitrophenyl)-glycinate.—A mixture of 1.8 Gm. (0.009 mole) of N-(*o*-nitrophenyl)-glycine, 15 ml. of absolute ethanol, and 1 ml. of concentrated sulfuric acid was refluxed for four hours. A portion of the alcohol was distilled, an excess of aqueous ammonia was added to the cooled residue, and the resulting solid was filtered and washed with dilute ammonia and water. Recrystallization from absolute ethanol gave 2.0 Gm. (97%) of fine yellow needles, m. p. 80–81°.

Anal.—Calcd. for $C_{10}H_{12}N_2O_4$: C, 53.56; H, 5.40. Found: C, 53.65; H, 5.65.

N-(m-Nitrophenyl)-glycine.—This compound was prepared by the same procedure as used for the *ortho* isomer. A 47% yield of yellow crystalline powder was obtained, m. p. 155–159° (lit. (14) m. p. 158–159°).

Ethyl N-(m-Nitrophenyl)-glycinate.—This ester was prepared by the same procedure as used for the *ortho* isomer. A 72% yield of golden platelets was obtained, m. p. 86–88° (lit. (15) m. p. 84°).

N-(p-Nitrophenyl)-glycine.—The same general procedure was used as for the *ortho* isomer. A 32% yield of yellow, crystalline powder was obtained, m. p. 230–232° (lit. (14) m. p. 225°).

Ethyl N-(p-Nitrophenyl)-glycinate.—The same procedure was used as for the *ortho* isomer. A 96% yield of light yellow needles was obtained, m. p. 138.5–140°.

Anal.—Calcd. for $C_{10}H_{12}N_2O_4$: C, 53.56; H, 5.40. Found: C, 53.33; H, 5.42.

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A Note on the Preliminary Phytochemical and Biological Study of *Lomatium Suksdorfii**

By FRANK A. PETTINATO†, LOUIS FISCHER, and NATHAN A. HALL

Lomatium Suksdorfii (Watson) Coult. and Rose, *Umbelliferae*, grows along the Columbia River in the state of Washington and is one of about 80 species of *Lomatium* [1] The roots of several species were used medicinally by the Gosiute Indians who called the plant "pia-a-na-tsu" or "great medicine" (2) The roots of two species have been reported to yield oils and extractives which were active antibacterials *in vitro* against numerous microorganisms (3–5) In the fuits of *L Suksdorfii*, Call (6) has found an oil and a crystalline substance which was an active spasmolytic

The work reported here was undertaken in preparation for a more exhaustive study of the components of the fruits and roots of the plant The plant material was collected in July 1956 near Klickitat, Washington, dried in air, and ground with a Wiley mill (1-mm screen) immediately before use The ground fruit and root were submitted to proximate analysis (see Table I) Both plant parts, when subjected to a glycoside extraction technique (7) produced a small amount of crystalline residue which was not further characterized All tests for alkaloids were negative

TABLE I —PROXIMATE ANALYSIS OF *L Suksdorfii*

	Root, %[a]	Fruit, %[a]
Moisture	7 8	7 0
Total ash	9 7	7 7
Acid insoluble ash	2 3	0 30
Reducing sugars (as invert sugar)	1 5	Trace
Sucrose	3 6	2 8
Starch	11 6	11 0
Nitrogen (Kjeldahl)	0 83	0 31
Protein (N × 6 25)	5 2	2 5

[a] Based on air dried weight

The ground root on steam distillation yielded 0 39% volatile oil and the ground fruit yielded 3 7% volatile oil Determination of the physical constants and chemical analysis of the fruit oil gave the results shown in Table II

TABLE II —PHYSICAL AND CHEMICAL PROPERTIES OF *L. Suksdorfii* OIL

Specific gravity, 25°/25°	0 8435
Refractive index, 23°	1 4808
Optical rotation, 25°	47 50
Acid number	0 42
Ester number	3 5
Ester number (after acetylation)	13 4
Esters (as bornyl acetate)	1 2%
Alcohols (as borneol)	3 7%
Aldehydes and ketones (by neutral sulfite method)	4 5%
Aldehydes and ketones (by hydroxylamine method, assuming average M W. of 150)	2 2%

The fruit oil was tested for antibacterial activity against growing cultures of *Micrococcus aureus* var. *pyogenes*, *Bacillus subtilis*, *Escherichia coli*, *Streptococcus pyogenes*, and *Proteus vulgaris* by the filter paper disk method of Vincent and Vincent (8) Simultaneous comparisons were made with cinnamon, eucalyptus, and rectified turpentine oils *L Suksdorfii* oil exhibited no significant activity against any of the organisms except *B subtilis* and *S pyogenes*, and in these cases the activity was no greater than the oils used for comparison

The volatile oil from the fruit was also screened *in vitro* for anthelmintic activity against *Ascaris lumbricoides* of swine by the Lamson and Brown procedure (9) When it was emulsified with 0 2% acacia, *L Suksdorfii* oil was ascaricidal in 4% concentration after a two-hour exposure period Much greater activity was shown by other anthelmintics used for comparison, hexylresorcinol (0 1%) was ascaricidal after a two-minute exposure and chenopodium oil was ascaricidal after a ten-minute exposure

Detailed investigation of the oil from the fruit will be reported in a subsequent paper

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[1] Considerable confusion regarding the genus *Lomatium* exists in the literature Although the term Lomatium is currently accepted, the genus has been variously designated as *Peucedanum*, *Cogswellia* and *Leptotaenia* (1) Specimens were identified by Dr A R Kruckeberg, University of Washington and Dr Lincoln Constance, University of California, whose assistance is gratefully acknowledged

The preparation of D-glucamine, N-(4-nitrophenyl)-D-glucamine, and N-(2,4-dinitrophenyl)-D-glucamine have already been reported (8). The preparation of N-(2-nitro-4,5-dichlorophenyl)-D-glucamine was carried out according to the method of Holly, *et al.* (9), and a 52% yield of product melting at 188–191° was obtained (Holly reported a m p of 193–195°).

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Anal.—Calcd. for $C_{10}H_{12}N_2O_4$: C, 53 56; H, 5.40. Found: C, 53 65; H, 5.65.

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TABLE I.—PROXIMATE ANALYSIS OF *L. Suksdorfii*

	Root, %[a]	Fruit, %[a]
Moisture	7.8	7.0
Total ash	9 7	7.7
Acid insoluble ash	2 3	0 30
Reducing sugars (as invert sugar)	1 5	Trace
Sucrose	3 6	2.8
Starch	11 6	11.0
Nitrogen (Kjeldahl)	0 83	0 31
Protein (N × 6.25)	5 2	2.5

[a] Based on air-dried weight.

The ground root on steam distillation yielded 0.39% volatile oil and the ground fruit yielded 3.7% volatile oil Determination of the physical constants and chemical analysis of the fruit oil gave the results shown in Table II.

TABLE II.—PHYSICAL AND CHEMICAL PROPERTIES OF *L. Suksdorfii* OIL

Specific gravity, 25°/25°	0.8435
Refractive index, 23°	1.4808
Optical rotation, 25°	47.50
Acid number	0 42
Ester number	3.5
Ester number (after acetylation)	13 4
Esters (as bornyl acetate)	1 2%
Alcohols (as borneol)	3 7%
Aldehydes and ketones (by neutral sulfite method)	4.5%
Aldehydes and ketones (by hydroxylamine method, assuming average M. W. of 150)	2.2%

The fruit oil was tested for antibacterial activity against growing cultures of *Micrococcus aureus* var. *pyogenes, Bacillus subtilis, Escherichia coli, Streptococcus pyogenes,* and *Proteus vulgaris* by the filter paper disk method of Vincent and Vincent (8). Simultaneous comparisons were made with cinnamon, eucalyptus, and rectified turpentine oils. *L. Suksdorfii* oil exhibited no significant activity against any of the organisms except *B. subtilis* and *S. pyogenes,* and in these cases the activity was no greater than the oils used for comparison.

The volatile oil from the fruit was also screened *in vitro* for anthelmintic activity against *Ascaris lumbricoides* of swine by the Lamson and Brown procedure (9). When it was emulsified with 0.2% acacia, *L. Suksdorfii* oil was ascaricidal in 4% concentration after a two-hour exposure period. Much greater activity was shown by other anthelminties used for comparison; hexylresorcinol (0.1%) was ascaricidal after a two-minute exposure and chenopodium oil was ascaricidal after a ten-minute exposure.

Detailed investigation of the oil from the fruit will be reported in a subsequent paper.

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* Received September 9, 1958, from the College of Pharmacy, University of Washington, Seattle 5

Supported in part by the Fund for Medical and Biological Research of the state of Washington

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[1] Considerable confusion regarding the genus *Lomatium* exists in the literature Although the term Lomatium is currently accepted, the genus has been variously designated as *Peucedanum*, *Cogswellia*, and *Leptotaenia* (1) Specimens were identified by Dr A E Kruckeberg, University of Washington and Dr Lincoln Constance, University of California, whose assistance is gratefully acknowledged.

A Note on the Use of Oxygen and Carbon Dioxide in Perfusion Fluids*

By G. H BRYAN and G R SHUCK

Reports of the perfusion of isolated mammalian hearts with artificial fluids is complicated by the use of various gases, as either oxygen alone or in combination with 1 or 5 per cent CO_2 (1) The two factors, the final oxygen concentration and pH, to which the tissue is exposed may lead to discrepancies in duplication of experimental results

In an extensive theoretical and applied treatment, Ling and Smith (2) have described the problem of pH control by gases in various perfusing fluids and have suggested a method of achieving a specific pH

The oxygen content has proved important in this laboratory and consequently it was of interest to find a procedure which would permit oxygen supersaturation and a final pH of 7 4

METHODS

Locke Ringer s U S P XV (3) solution was used Ten liters were freshly prepared each time the solution was needed To facilitate electrical stirring, pH measurement, and sample withdrawal, a 15 L battery jar was used as the container Oxygen and 5% carbon dioxide in oxygen were delivered through gas dispersion tubes (coarse) submerged to 20 cm and mounted on the side of the jar Gas flowed through a 5 mm rubber tube under 67 mm of mercury gauge pressure This corresponds roughly to a vigorous but not turbulent flow Oxygen determinations were made by the Rideal Stewart modification of the Winkler method (4) So that monitoring of pH could be continual, four foot extensions were spliced into the electrode systems of a Beckman pH meter, Model H2

RESULTS

Twelve one hour series of oxygen determinations at ten minute intervals from the onset of oxygen gassing revealed that a near maximum saturation was reached in twenty minutes This was found with Locke Ringer's solution at 37, 26, and 10° After the gas flow and stirring was stopped, equilibration with the air did not affect the oxygen content of Locke Ringer's solution gassed at 26 and 10° for approximately two hours Therefore, with this fixed oxygen flow, a lesser time of gassing does not allow saturation to be reached and a greater time wastes oxygen

If a physiological pH is considered to be 7 4, then it was of interest to monitor the pH resulting from oxygen gassing From a starting value of 7 9 the pH was 8 6 at the end of twenty minutes Therefore, the condition of pH 7 4 cannot be met with oxygen gassing of Locke Ringer's solution This is in general agreement with Ling and Smith (2) for other fluids and for the sodium bicarbonate content Gassing in the same manner with 5% carbon dioxide in oxygen resulted in a pH shift from a starting value of 7 8 to 6 7 after twenty minutes of gassing Therefore, the condition of pH 7 4 cannot be met with 5% carbon dioxide in oxygen gassing

As a result of these findings with Locke Ringer s solution it was necessary to gas with oxygen for twenty minutes to obtain saturation, then shift to gassing with 5% carbon dioxide in oxygen until a pH of 7 4 had been obtained Over and undershooting could be reversed with the appropriate gas

Variations exist in the ultimate delivery of perfusion fluids Tissue may be placed in the container in which the fluid was prepared or the fluid may be moved to the tissue through a series of glass or rubber tubes There also exists the possibility of a temperature gradient from a stock container to the tissue In this instance, it was desired that the fluid be pumped from a stock container to a reservoir 90 cm above the table top, allowed to flow through a constant temperature bath (1,000 watt heater) at 37°, and then be delivered to an isolated heart suspended 125 cm below the reservoir Oxygen values found at the start and finish of this route and at three different starting temperatures are shown in Table I From Table I, it may be seen that there is a gain in oxygen at the delivery end of the system if the initial gassing is conducted at temperatures lower than 37° Whether the gain at lower temperature is important or not will depend upon tissue requirement

As a practical matter, storage of gassed solutions for twelve hours in filled, rubber stoppered containers at ice box temperatures has been satisfactory

SUMMARY

It is recommended that perfusion fluids of the Locke Ringer type be gassed with oxygen at temperatures lower than 37° for twenty minutes and the pH adjusted by gassing with 5% carbon dioxide in oxygen The solution may then be stored or used within a two hour period

Table I — Solubility of Oxygen[a] in Locke-Ringer's Solution at Various Temperatures Under 673 mm of Hg Pressure

Dissolved oxygen in parts per million by weight

Temp °C	A	B	C
37	22 2	21 0	21 0
26	31 0	29 0	25 0
10	42 4	38 0	28 0

A—O_2 Content after twenty minutes of gassing
B—O_2 Content after additional gassing with 5% CO_2 in O_2
C—O_2 Content after pumping solution from stock container and through a perfusion system
[a] The above values are approximately four times the equilibrium value listed by Griffin (4) due to supersaturation

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* Received April 18 1957 from the School of Pharmacy, Montana State University Missoula

A Note on the Stability of Vitamin B_{12} in the Presence of Thiochrome*

By LOUIS J. RAVIN and ROBERT F. DOERGE

In a recent review article Scheindlin (1) discusses the chemical interactions of the water-soluble vitamins. He points out that while several studies have been published (2–6) concerning the stability of vitamin B_{12} in the presence of thiamine and nicotinamide, much remains to be learned about the reaction between thiamine and nicotinamide, as well as the reaction between thiamine and vitamin B_{12}. In addition to the references cited by Scheindlin (1), additional information on this problem has been reported by Gambier and Rahn (7, 8). Several of these studies have implicated the thiazole moiety of thiamine in the breakdown of vitamin B_{12}, however, no mention has been made concerning the possible role of thiochrome. This note presents some preliminary data regarding the possible interaction of thiochrome, an oxidation product of thiamine, with vitamin B_{12} in aqueous buffered solution.

EXPERIMENTAL

Thiochrome obtained from Bios Laboratories Inc. and cyanocobalamin U. S. P. (crystalline vitamin B_{12}, Merck and Co.) were used in these studies.

Solutions containing 25 μg. of crystalline vitamin B_{12} and 8.5 mg. of thiochrome[1] in each 5 ml. were prepared and adjusted to pH 4.0. A control solution containing 25 μg. of crystalline vitamin B_{12} was also prepared. The finished solutions were filtered through a Selas[2] unglazed porcelain candle, 02 porosity, and packaged into 1-oz. amber bottles. Samples were set down at RT, 45, 60, and 85° and assayed periodically for vitamin B_{12} by the U. S. P. microbiological method (9).

DISCUSSION

The assay data obtained in this study are summarized in Table I.

Table I.—Stability of Crystalline Vitamin B_{12} in Aqueous Buffered Solution Containing Thiochrome[a]

Storage Condition	Storage Time	Solution Containing Thiochrome, μg./5 ml.	Control, μg./5 ml
Original	.....	28	28
85°C.	8 hours	28	28
	16 hours	26	27
	24 hours	26	26
RT	3 weeks	29	29
45°C.	3 weeks	27	30
60°C.	3 weeks	29	29
RT	7 weeks	29	26
45°C.	7 weeks	27	24
60°C.	7 weeks	25	24
RT	3 months	27	28
45°C.	3 months	27	26
60°C.	3 months	23	22

[a] 8.5 mg./5 ml., equivalent to 11 mg./5 ml. thiamine hydrochloride.

The above data clearly indicate that thiochrome is not responsible for accelerating the decomposition of vitamin B_{12} in an aqueous buffered solution under these experimental conditions. Additional studies concerning the interaction between thiamine and vitamin B_{12} which also include the possible reaction with thiamine decomposition products are in progress. A complete report on the results of these studies will be forthcoming.

* Received September 20, 1958, from the Research and Development Division of Smith Kline and French Laboratories, Philadelphia 1, Pa.

[1] Equivalent to 11 mg./5 ml. thiamine hydrochloride.
[2] Selas Corporation of America, Philadelphia, Pa.

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Book Notices

Handbook of Toxicology. Vol. IV. Tranquilizers. Edited by Rudolph M. Grebe. W. B. Saunders Co., West Washington Square, Philadelphia, Pa., 1959. viii + 120 pp. Paperbound. Price $4.

This handbook is the eighth of a continuing series of publications, each containing information, chiefly tabular, in one or more of the biological sciences. All of the volumes in the series have been prepared under the general direction of the Committee on the Handbook of Biological Data, Division of Biology and Agriculture, National Academy of Sciences National Research Council. This latest volume furnishes, in outline form, pertinent data on 26 tranquilizers which have been used as psychopharmacological agents. In general, the information about each of the drugs in this class includes the nonproprietary name, the trademark, the name of the manufacturer, the molecular formula and weight, the structure, a brief description of the physical and chemical properties, the pharmacology, certain clinical data, the toxicity, and the mode and site of action. The book is documented with the more important references to the original literature. The book is well designed to furnish information in summary form on the principal tranquilizers now being used.

Ubersicht der gebrauchlichen und neueren Arzneimittel By E BERNOULLI and H LEHMANN Benno Schwabe & Co, Basel, Switzerland, 1959 U S Representative, Intercontinental Medical Book Corp, New York xlviii + 555 pp 12 x 18 cm Price $5 75

This relatively small book contains enormous amounts of information in outline form, concerning newer medicinal chemicals as well as many of the older, but still widely used drugs The information presented is logically classified according to the therapeutic categories of the various drugs discussed The book is particularly helpful in locating information on the nature of German and certain other European trademark names and nonproprietary names of drugs Chemical and physical properties of the drugs covered are usually presented, and a list of available dosage forms are often given The book is provided with an unusually complete index which facilitates locating needed information easily and promptly

Hagers Handbuch der Pharmazeutischen Praxis In 2 vols Edited by WALTER KERN Springer-Verlag, Berlin, 1958 xvi + 2,544 pp 16 5 x 24 cm Price DM 224

A complete revision of this well known reference book on the practice of pharmacy is now available in two volumes, the first covering the alphabet A–H and the second I–Z The two volumes are paged consecutively, and the second volume contains the index for both The new edition continues to be a comprehensive and thoroughly reliable reference for many pharmaceutical fields of endeavor A more complete review of the new edition of Hager appears in THIS JOURNAL, *Pract Pharm Ed* 20, 342(1959)

The Profession of Pharmacy By RICHARD A. DENO, THOMAS D ROWE, and DONALD C BRODIE J B Lippincott Co, East Washington Square, Philadelphia, Pa, 1959 xii + 256 pp 18 x 25 5 cm Price $6 50

This introductory textbook is unique in character and different from any other textbook that has ever been written It provides the beginning student in pharmacy with a broad coverage of the profession including its historical development, ethical standards, organizations, literature, and current problems Its organization covers the major fields of pharmaceutical practice including pharmaceutical education, retail pharmacy, pharmaceutical research, manufacturing pharmacy, promotion and distribution of drugs, hospital pharmacy, development of standards for drugs, and activities involving pharmaceutical legislation Each chapter is followed by provocative study questions and a list of selected references for additional reading The scope of the book can best be visualized from the titles of the fifteen chapters which are Pharmacy and other health professions, Birth of a drug, Pharmaceutical education, Roots of retail pharmacy, Modern retail practice, Organizations and periodicals of retail pharmacy, Nature and climate of science, Pharmaceutical research, The pharmaceutical industry, Production and quality control, Promotion and distribution of drugs, Hospital pharmacy, Pharmacopeias and other standards, Legal regulation, Pharmacy—past and future Interest in the text is enhanced by the inclusion of 85 well chosen illustrations of the subject matter discussed The design of the book and the presentation of the subject matter are excellent throughout, and reflect the thought and care of the authors that must have gone into its preparation It should be well received, and probably will be adopted as a standard textbook in many colleges of pharmacy

Précis de Chimie Générale et de Chimie Minérale Book I By L DOMANGE Masson et Cie, boulevard Saint Germain, Paris 6ᵉ, France, 1959 305 pp 16 x 21 5 cm Price 2,200 fr

This book (in French) is a concise review of general and inorganic chemistry intended for use as a textbook for one semester in a course for beginners It is designed as part of a series of textbooks for pharmacy students

Principles of Microbiology 2nd ed By WALTER H KRUEGER and KARL R JOHANSSON W B Saunders Co, West Washington Square, Philadelphia 5, Pa 1959 xxiv + 563 pp 15 5 x 23 5 cm Price $6 75

The second edition of this textbook has been completely revised by the inclusion of new developments and points of view in the science of microbiology The general organization of the previous edition is a retained, but a substantial amount of new material and several new illustrations have been introduced The book is intended primarily as a textbook for an introductory survey course in general microbiology for college students

Index of Medicines Produced in Israel By J ALADJEMOFF F Pinczower, Tel-Aviv, Israel, 1953 Available in the U S from J Pincover, 602 West 157th St, New York 32, N Y, 492 pp 12 x 16 5 cm

This book lists alphabetically drugs produced in Israel, including the dosage forms, indications for use, dosage, and other pertinent information The list indicates that the pharmaceutical and chemical industry in Israel is growing and developing rapidly This may be due in part to restrictions on imports of drugs produced in other countries The book is designed to assist physicians in choosing the proper preparation, ascertaining its dose, and the directions for its administration

Selected Papers from the Institute of Cancer Research Royal Cancer Hospital and from the Royal Marsden Hospital Vol II Lund Humphries, London, 1958 xix + 928 pp 19 5 x 26 5 cm

A collection of reprints of ninety eight papers relating to chemical, pharmacological, and clinical aspects of cancer published in scientific journals during 1956 This compilation should serve as a very great convenience to any worker in cancer research by having available in one volume such a large number of significant published reports

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII | *AUGUST 1959* | NUMBER 8

Studies on the Adsorption of Odorous Materials I*

Surface Potential Changes of Solid and Liquid Adsorbing Surfaces

By J. O. KOPPLIN†, J. R. EATON, and J. E. CHRISTIAN

The measurement and classification of odors has been greatly handicapped by the lack of an objective instrument for the measurement of odor. This paper describes experiments in which the detection of odorous material in low concentrations in an air stream was made by measuring the change in surface potential of an adsorbing surface in contact with the air stream. Various solid and liquid surfaces were used including water. The effect of the presence of water vapor in the air stream on the changes in the surface potential of various surfaces was studied as well as the effect of increasing the temperature of the adsorbing surface. These tests indicate the possible use of surface phenomena for the detection of odorous material in the atmosphere.

In an effort to gain background information for the development of an objective instrument for the measurement of odor, various surface properties of many materials have been investigated to determine whether and how they are affected by the presence of odorous materials in the vapor or gaseous state. In 1950 Tanyolac and Eaton (1) showed that the surface tension of various liquids could be used to detect the presence of, and to gain identifying information on, certain vapors from a saturated atmosphere. Following this, various odors and the deodorizing effects of certain materials were studied by the School of Pharmacy at Purdue University using surface tension measurements (2, 3). Chapman and Eaton (4) in 1955 reported that very small amounts of certain vapors in an air stream could be detected and partially identified by the measurement of changes in the surface potential of different solid surfaces. This paper outlines the continuation of this investigation at Purdue University.

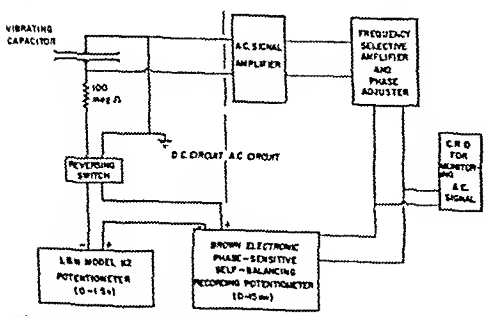

Fig. 1.—Schematic diagram of measuring circuit.

EQUIPMENT

Surface potential cannot be directly measured with the surface exposed to the atmosphere at normal pressure. However, changes in surface potential can be determined by measuring the contact potential difference between two surfaces, using as a reference one surface the potential of which does not vary. The Kelvin method for measuring contact potential differences, as modified by Zisman (5), was used in this investigation. A schematic diagram of the electrical circuit is shown in Fig. 1. The apparatus consisted of two parallel plates which formed the vibrating capacitor. The upper plate or electrode was desensitized with a coating of paraffin wax or other material so that little or no adsorption took place on the upper surface from the air stream which passed between the plates. The upper electrode was grounded and was vibrated by a small electromagnet carrying alternating current. The lower surface was always the surface upon which adsorption was

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† [illegible] ineering Department, Uni[illegible]

studied This electrode was insulated by a teflon bushing and was resistance-capacitance coupled to the amplifier used to amplify the alternating signal from the vibrating capacitor Semiautomatic null-balancing was provided by a phase sensitive self-balancing recording potentiometer in series with a manual potentiometer and the vibrating capacitor When liquid rather than solid adsorbing surfaces were used, the lower electrode of the vibrating capacitor was replaced with a shallow metal dish holding approximately 8 ml of liquid The polarity of the contact potential was taken as positive when the field due to the contact potential difference alone was directed upward between the two surfaces

The air used in this research was purchased from a commercial vendor and supplied in high-pressure tanks This was done to secure a more uniform air stream free from day to day variations, and also to obtain a constant pressure air supply The air was released through a pressure regulating valve and passed through a filter composed of charcoal and anhydrous calcium chloride and calcium sulfate From the filter, the entire air stream, or a percentage of it, could be passed through a bath of triply distilled water which was used to control the relative humidity of the stream Three contaminant bottles could be connected to the system at one time through intervening teflon stopcocks Contaminant vapor was injected into the air stream through displacement of the vapor in the contaminant bottle by mercury flowing in at a measured rate A schematic air flow diagram is shown in Fig 2

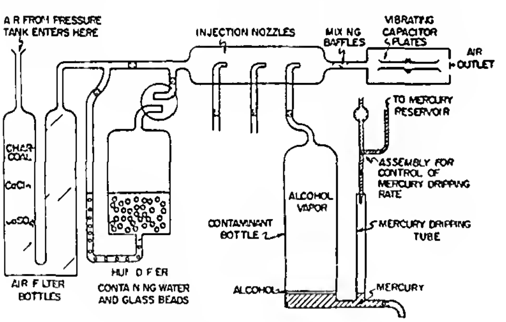

Fig 2 —Schematic air flow diagram

The general procedure used was to pass the air stream through a test cell containing the two plates or surfaces of the vibrating capacitor electrometer at a rate of 100 liters per hour The surface potential of the sensitive surface was monitored for a minimum period of five minutes to make sure there was neither excessive drift nor instability in the surface potential Contaminant vapor was then injected into the air stream at a specific rate, and the resulting change in surface potential was recorded until the surface potential again became stable, indicating equilibrium conditions for the adsorption process At this time injection of the contaminant vapor into the air stream was stopped, and the change in surface potential was recorded until the potential again assumed a stable value Generally, the surface potential, after an injection of contaminant vapor into the air stream, returned to or very close to the value it had prior to the contaminant vapor injection In most cases not more than three injections of the same or different contaminant vapors were made using one particular surface, as a given surface showed a certain loss of sensitivity with repeated injections of contaminant vapor and prolonged exposure to the air stream Surface sensitivity for a particular air stream contaminant, as used in this paper, is measured by the ratio of the change in surface potential expressed in millivolts (mv) to the contaminant vapor concentration in the air stream expressed in parts per million by volume (p p m)

EXPERIMENTATION AND DISCUSSION

Many different surfaces have been used in this investigation including gold, aluminum, charcoal, powdered zinc, powdered tellurium, mercury, and water The most sensitive surface to many contaminants in low concentrations in the air stream was a gold plate dusted with finely powdered tellurium With this surface the change in potential was often in the range of 20 to 80 mv depending upon the contaminant vapor and its concentration

As far as the detection of odorous material in an air stream was concerned, the chief disadvantage of all *the solid surfaces used was their loss in sensitivity to* contaminant vapor as the humidity of the air stream was increased It was found that a dusted tellurium surface has a sensitivity of only 20% to ethyl alcohol vapor in an air stream of 30% relative humidity as compared to its sensitivity to ethyl alcohol vapor when carried by a dry air stream This, along with the thought that if the process of olfaction is due wholly or in part to adsorption, the adsorbing surface would be most likely a liquid, was the reasoning behind the investigation of liquid adsorbing surfaces

The outstanding advantage of a distilled water surface was that it showed no loss in sensitivity to alcohol vapor when the relative humidity of the air stream was increased to 70% and higher This was noticeably different from all solid surfaces used and was thought to be due to the fact that the air just immediately above the water surface had a high water vapor content caused by evaporation from the surface, regardless of the humidity of the air stream Because adsorption and desorption of water molecules was continually taking place, changes in the humidity of the air stream had a negligible effect upon the surface potential of a water surface

Three members of the homologous alcohol series—ethyl, normal propyl, and normal butyl—were adsorbed one at a time upon a powdered tellurium surface and also upon a water surface It was found that the overall pattern of the changes in the surface potentials for the two surfaces, shown in Figs 3 and 4, was very similar The change in surface potential of the tellurium surface was greater for the same amount of ethyl alcohol vapor in the air stream than that of the water surface However, when comparisons are made between the tellurium and water surfaces, it should be noted that the adsorption of the alcohol vapor on the tellurium surface was from a dry air stream while the adsorption on the water surface was from a humid air stream

The effect of varying the temperature of the adsorbing surface was investigated as a means of distinguishing between various air stream contaminants This particular phase of the investigation was based on the theory that different types of adsorbates are held to the surface by different binding energies For an adsorbed molecule to desorb from

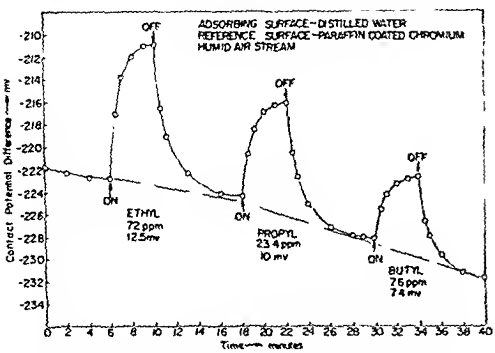

Fig. 3.—Water surface potential change due to adsorption of three different alcohol vapors.

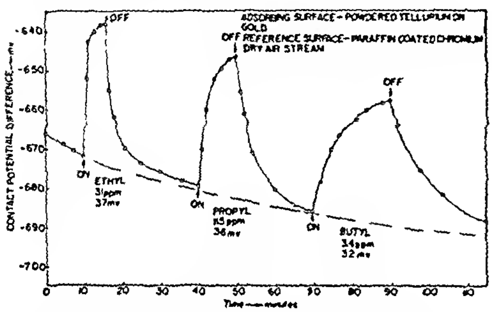

Fig. 4.—Tellurium surface potential changes due to adsorption of three different alcohol vapors.

the surface, it must be supplied with an energy equal to or greater than its binding energy, and a usual source for this escape energy is the thermal energy of the adsorbent. Thus, as the temperature of the adsorbent is increased, the greater is the chance for any given molecule to receive sufficient energy to desorb. For this reason, with constant pressure but increasing temperature, the amount of material on the surface at any one time would be expected to be less. Therefore the possibility exists that for a given surface and type of adsorbate, a unique temperature exists at which the amount adsorbed on the surface would be insufficient to cause a measurable change in the surface potential.

For the investigation of temperature dependence, it was necessary to find a surface which was sensitive to alcohol and other air stream contaminants, and one which after being heated in the presence of a pure air stream regained its original sensitivity to air stream contamination upon return to room temperature. This was necessary so that any reduced sensitivity of the surface to contaminant vapor at a higher temperature could be attributed to the increased temperature and not to a change in the surface itself due to its being heated in an air stream. To test this property of a surface, its sensitivity to a contaminant in the air stream was first measured at room temperature. Then the surface was heated in the air stream to 180° F. and allowed to cool back to room temperature. Following this, the surface was again exposed to the same contaminant at the same concentration as the original exposure and its sensitivity was again measured to determine whether it had changed. A number of solid surfaces were tested in this manner including aluminum and tellurium, but in each case their room temperature sensitivity was greatly reduced by the heating process. It was found that a surface made up of powdered wood charcoal on an aluminum plate previously heat-treated had sufficient sensitivity to alcohol vapors in the air stream, and its sensitivity at room temperature was not reduced due to its being heated in a pure air stream. This surface was adopted for this phase of the investigation.

The first tests were run using two different concentrations of ethyl alcohol vapor and one of butyl alcohol. These tests were made by first measuring the change in the surface potential at room temperature with a given concentration of the alcohol vapor in the air stream. Following this, the change in surface potential was again measured using the same vapor concentration; first, with the temperature of the adsorbing surface increased above room temperature and second, with the adsorbing surface again at room temperature. The results of a typical test of this type are shown in Fig. 5. Tests were run with the temperature of the lower plate ranging from 78 to 180° F.

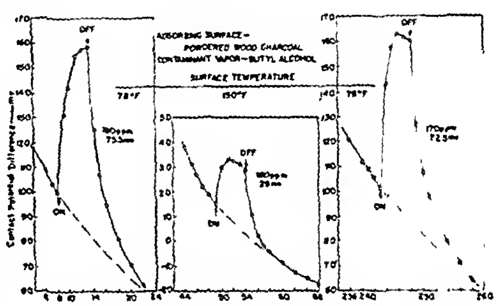

Fig. 5.—Surface potential change *vs.* time at two different temperatures.

A new surface was used for each test, which involved three equal injections of alcohol vapor into the air stream. The surfaces were all made up in the same manner using powdered wood charcoal on an aluminum plate; however, no two surfaces were alike, and their room temperature sensitivities did vary up to 20%. In order to compare the various tests, the sensitivity at the increased temperature was expressed as a percentage of the room temperature sensitivity for each individual surface used. This was done to minimize the differences in the sensitivities of the various surfaces. For calculating the per cent sensitivity at a given temperature, the room temperature sensitivity following the check at an elevated temperature was taken as 100%. For the test shown in Fig. 5 the sensitivity of the surface at 150° F. to butyl alcohol vapor was 38% of the room temperature sensitivity.

Figure 6 shows the per cent sensitivity plotted against the temperature of the adsorbing or lower surface for two concentrations of ethyl and one of butyl alcohol vapors in the air stream. The per cent sensitivity at the highest temperature reached was between 20 and 30% for both alcohol vapors. For intermediate temperatures, the differences between the per cent sensitivity for the two alcohol vapors were not appreciable and varied with the concentration of the vapor in the air stream.

Two other contaminant vapors were tested—butyl chloride and diethyl ketone. A similar reduc[illegible] the sensitivity with increased temperature w[illegible]

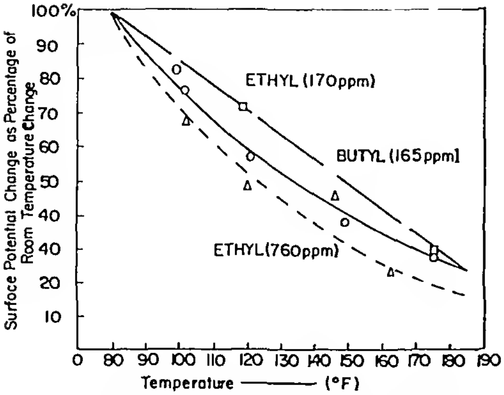

Fig 6—Reduction in surface potential change due to increased temperature

using these two vapors However, a significant difference was found in that the given concentration of butyl chloride produced no significant change in the surface potential when the adsorbing surface was heated to 165° F Two different checks were made at 165° F, in neither case did the butyl chloride vapor cause a change in surface potential and in each case, after the surface had cooled to room temperature, a definite sensitivity to butyl chloride vapor was found This same pattern was established using diethyl ketone but the "cut off" temperature was 180° F

These tests were very limited and more work needs to be done however, the initial results indicate the possibility of distinguishing between adsorbates of different chemical classification by determining the temperature range over which various vapors cause a measurable change in the surface potential of a stable adsorbing surface

The tests with the alcohol vapors indicated that the highest temperature at which a change in surface potential might be detected was independent of the vapor concentration in the air stream The fact that small increases in temperature caused a greater reduction in the per cent change in surface potential for the higher ethyl alcohol vapor concentration apparently indicates a lower binding energy for the additional molecules adsorbed because of the higher vapor concentration Blench and Garner (6) found that the heat evolved by oxygen on charcoal was much higher for the first portions adsorbed than for latter portions Thus, if the first portion of ethyl alcohol vapor adsorbed at either of the two concentrations used evolved the same amount of heat, it would be expected that the maximum temperature at which a change in surface potential occurred would be the same regardless of vapor concentration This would also be necessary to distinguish between various classes of vapors by this type of test if the vapor concentrations were unknown

Measurement of the actual amount of material adsorbed or responsible for a given change in surface potential was considered a desirable experiment in connection with this investigation Because the amounts of adsorbed material were extremely small, the use of radioactive isotopes was considered the most feasible method for measurement of the quantity of adsorbed material Initial experimental work was done using carbon-14 labeled ethyl alcohol Two methods for detecting carbon 14 labeled molecules on a water surface were investigated The first method employed a thin window carbon-14 counter placed immediately over the water surface upon which the labeled alcohol had been adsorbed The second method involved liquid scintillation counting (7), using the water which formed the adsorbent surface as part of the liquid scintillation sample Each method has inherent errors for which correction should be made The major errors are due to the rapid desorption rate of the adsorbed molecules and to the solubility in water of the shorter chain alcohols The initial experimental results, however, definitely established the fact that the amounts of material responsible for measurable changes in the surface potential can be measured using radioactive tracer techniques Additional work is being carried out in this area with current efforts being directed toward the simultaneous measurement of the change in surface potential and determination of the amount of adsorbed material responsible for the change

SUMMARY

The general conclusions concerning this research project are that

1 Measurable changes in the surface potential of many surfaces are produced by the adsorption of odorous material from the atmosphere

2 The surface potential of a distilled water surface is sensitive to the presence of many different organic vapors carried by an air stream, and this sensitivity is largely independent of the relative humidity of the air stream

3 There is a possibility that vapors of different chemical classification can be distinguished by determining the adsorbing surface temperature just sufficient to prevent measurable changes in surface potential

4 The number of adsorbed molecules causing a change in surface potential can be measured by the use of radioactive tracer techniques

The results of these tests further set forth the possible use of surface phenomena for the detection of odorous atmospheric contamination

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A Kinetic Study of the Hydrolysis of Methyl DL-α-Phenyl-2-piperidylacetate*

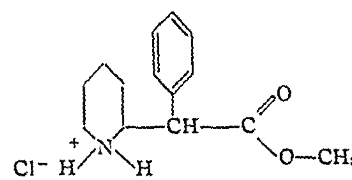
By SHELDON SIEGEL†, LEON LACHMAN‡, and LOUIS MALSPEIS

The kinetics of the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate[1] in aqueous buffers were studied over the pH range 1.1 to 6.1. The hydrolysis appears to be catalyzed specifically by hydronium and hydroxyl ions. The resulting catalytic catenary afforded a pH minimum of 2.86 at 80°. The pseudo first- and second-order velocity constants for hydronium and hydroxyl ion catalysis were determined, and the temperature dependency of the reaction was studied. The influence of ionic strength on the hydronium and hydroxyl ion catalysis was investigated. The structural characteristics of the molecule contributing to the isocatalytic point are discussed.

The catalytic catenary of ester hydrolysis has been the subject of considerable investigation (1–3) but relatively few determinations of the catenary have been reported for cationic esters of aliphatic acids (4–8). The catenary can be calculated from the kinetic data of acid and base-catalyzed hydrolysis of esters having a permanent formal positive charge, e. g., a quaternary nitrogen (5, 6, 9–12). However, if the presence of the positive center in the molecule depends upon the hydrogen ion concentration of the solution, as occurs with substituents which are primary, secondary, and tertiary amines, the catenary cannot be determined from the second-order specific velocity constants due to hydronium ion and hydroxyl ion catalysis which are cited in the literature, since the former constant applies to hydronium ion attack on the protonated ester whereas the latter constant applies to the reaction of hydroxyl ion with the nonionic ester (13, 14). Consequently, little information is available regarding the nature of the hydrolytic catenary of a cationic ester.

The isocatalytic point of the catalytic catenary for a reaction specifically catalyzed both by hydronium and hydroxyl ions corresponds to that hydronium ion concentration at which the partial velocities due to hydronium and hydroxyl ion catalysis are equal (1). It is a composite function of the entropies and heats of activation of the hydronium and hydroxyl ion catalyses and is influenced by both steric and electronic effects of substituent groups on the substrate. Moreover, at the isocatalytic point overall reaction velocity is minimal and stability of the substrate is maximal.

The present paper reports a preliminary kinetic study of the hydrolysis of the central nervous system stimulant, methyl DL-α-phenyl-2-piperidylacetate hydrochloride (I).



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The pKa of this ester is approximately 8.8, and therefore, it is fully protonated in a solution whose pH is below 6.8. This study deals with the hydroxyl and hydronium ion—catalyzed hydrolysis in the pH range of 1.1–6.2 in order to estimate the apparent isocatalytic point of the hydrolytic catenary. Another objective of this investigation is to compare the apparent isocatalytic points, frequency factors, and energies of activation of other cationic esters with the values found for methyl DL-α-2-piperidylacetate hydrochloride.

EXPERIMENTAL

Reagents.—Methyl DL-α-phenyl-2-piperidylacetate hydrochloride, Ciba, recrystallized from ethanol and dried in vacuum, m. p. 204.5–205.5° (lit. (15) 204–208°). The reagents used as buffers and in the analytical procedures were of analytical grade. Potassium hydrogen phthalate, potassium dihydrogen phosphate, and disodium hydrogen phosphate were dried at 110° for two hours prior to use. Eastman Kodak Company white label cyclohexane was used without further purification.

pH and Buffers Solutions.—The pH values reported are all "apparent" values inasmuch as they are subject to liquid-junction potential error. A Beckman model G pH meter provided with a fiber type saturated calomel electrode and a "General Purpose" glass electrode was used to determine the pH of samples at room temperature. Because the temperature compensation of this instrument do

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not extend beyond 40°, the Beckman model H-2 pH meter was used to measure "apparent" pH values at elevated temperatures The pH meters were standardized in the conventional manner with standard phthalate (pH 4 01 at 25°) and standard phosphate (pH 7 00 at 25°) buffers All standardizations were carried out at the temperature of the solution being measured The pH values of the standard buffers at elevated temperatures were those recorded by Britton (16) The pH values at elevated temperatures appear to be reproducible within ±0 02 pH unit Temperature correction at elevated temperatures are in general accord with those of Bates (17) The concentration of the buffer used was determined by its capacity to maintain a constant hydrogen ion activity throughout the reaction The pH of the hydrolysis mixture was determined before and after equilibration at bath temperature as well as subsequent to each kinetic run

The buffers used in this study are listed in Table I The ionic strength of the final solutions employed in the kinetic runs recorded in Table I were not corrected for expansion of solvent volume at elevated temperatures They were calculated from the expression $\mu = {}^1/_n Cz^2$ in which μ is the ionic strength, C is the concentration in moles per liter, and z is the ionic charge pH 1 1, μ = 0 106, pH 1 3, μ = 0 106 pH 2 0, μ = 0 106, pH 2 8, μ = 0 056, pH 3 2, μ = 0 056, pH 4 2, μ = 0 107, pH 5 1, μ = 0 200, pH 6 1, μ = 0 569

Temperature Control.—The thermostats employed were maintained at 50, 65, 80, and 95° The 50 and 65° baths were capable of maintaining a constant temperature of about ±0 02°, the 80° bath maintained a constant temperature of about ±0 03°, while the 95° bath was capable of no better than ± 0 06° The bath temperatures were determined with a thermometer certified by the National Bureau of Standards

ANALYTICAL METHODS

Iron (III) Hydroxamic Acid Colorimetric Method.—For the purpose of the present study, it was desirable to develop an analytical technique which would permit the differentiation of the undegraded methyl DL-α-phenyl-2-piperidylacetate from its degradation products The assay procedure generally used throughout this paper was an adaptation of the reaction of carboxylic esters and hydroxylamine

Essentially, this method of assay is based on the conversion of the ester into alkali salts of hydroxamic acids This is subsequently converted to the free hydroxamic acid by acidification and reacted with trivalent iron to form a red-colored complex The colored complex shows an absorption spectrum in the visible range and the intensity of the color is related to the concentration of the ester

The absorption characteristics of the iron hydroxamic acid complex of the ester are presented in Fig 1 It can be seen from this figure that the extinction of the blank falls off quite sharply in the range of optimum absorption for the ferric hydroxamic acid complex of methyl DL α-phenyl-2-piperidylacetate The complex exhibits a broad maximum at 500 mμ However, at this wave length the blank solution also shows a significant absorp-

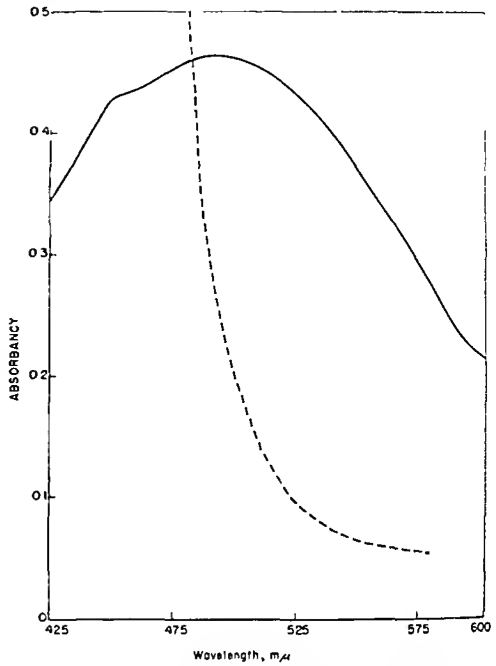


Fig 1 —Ultraviolet absorption spectra of ———, methyl DL α-phenyl 2 piperidylacethydroxamic acid-ferric ion complex, - - - - -, ferric chloride blank solution

tion, while at a wave-length from 520–540 mμ, the absorption of the blank is substantially reduced Since at a wave-length from 520–540 mμ the complex still exhibits absorption close to maximal, it was decided to use a wave-length of 530 mμ in this study Furthermore, by employing this higher wave length for the assay, it was possible to use higher concentrations of ferric chloride to form the colored complex Through the use of these higher concentrations of ferric chloride the stability of the colored complex formed is increased and thereby more suitable

The Beckman model DU spectrophotometer was employed to measure the absorbance of the solutions

It was further found that the rate of reaction of ester and hydroxylamine as well as the final color intensity of the complex were pH dependent Hestrin (18) reported that the color intensity was essentially independent of pH between pH values of 1 0 and 1 4 The final pH of the solution of the colored complex was 0 55 Attempts to decrease the hydrogen ion concentration resulted in a precipitation of ferric hydroxide due to the high ferric ion content

Extraction Method.—The ultraviolet spectra of the ester in cyclohexane are described in Fig 2 The cyclohexane solutions are extracts of the hydrolysis mixture which had been made alkaline at the time of extraction Spectrum A represents the unhydrolyzed ester while spectrum B applies to the degradation which has proceeded beyond one half-life These results suggested that the hydrolysis could be followed by determining the decrease in extinction at 260 mμ It was established that the sodium salt of methyl DL-α-phenyl 2 piperidyl-

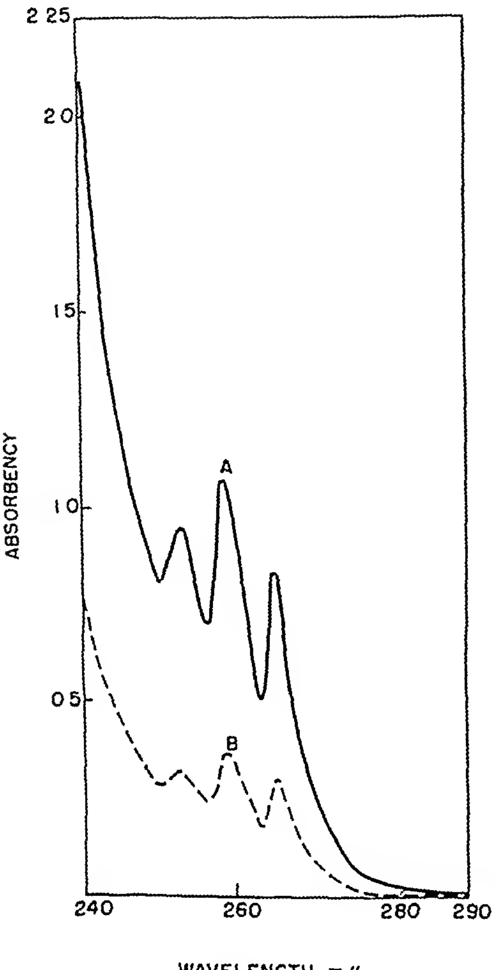


Fig 2 —A plot of the ultraviolet absorption spectra of methyl DL-α-phenyl-2-piperidylacetate in cyclohexane Curve A represents the absorption spectrum for the undegraded ester, while Curve B denotes the change after hydrolytic degradation

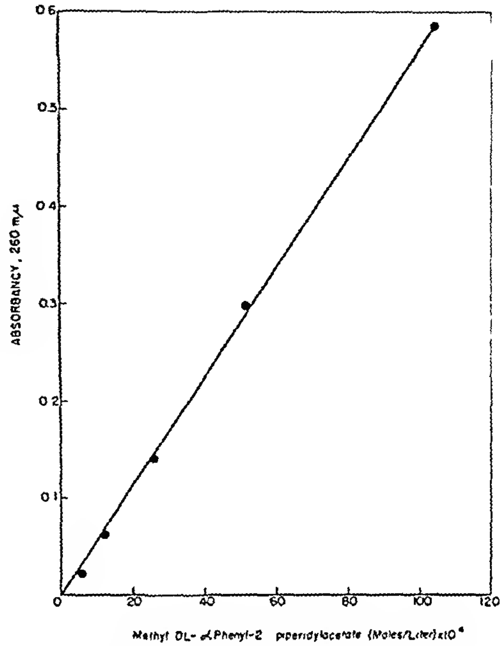


Fig 3 —Relationship between methyl DL-α-phenyl-2-piperidylacetate concentration and absorbance in cyclohexane

acetate does not exhibit any significant solubility in cyclohexane as evidenced by a lack of extinction at 260 mμ

The procedure adopted involved placing a 2 00-ml sample of the hydrolysis mixture which had an initial known concentration of approximately 0 008 *M* ester in a ground glass-stoppered tube and pipetting onto this sample exactly 5 00 ml of cyclohexane One ml of 0 50 *M* sodium hydroxide was then added, the aqueous solution extracted by thoroughly shaking the tube, and the tube was then centrifuged for several minutes A portion of the cyclohexane phase was then withdrawn and the absorbance determined at 260 mμ, using the smallest slit width possible, 0 7–0 8 mm Figure 3 describes the Beer's law relationship when dilutions were performed on the aqueous solution of the ester and each solution was extracted according to the described procedure

Excellent correlation was found between the results obtained for the colorimetric assay method and the extraction procedure In this study, results obtained by the extraction method are noted, and if no mention is made, the colorimetric assay procedure was used

Kinetic Procedure.—A 300-mg sample of recrystallized methyl DL-α-phenyl-2-piperidylacetate was quantitatively weighed and transferred to a 200-ml volumetric flask and brought up to volume with buffer solution The solution was then injected into U S P XV type I ampuls of 10 ml capacity, sealed, and immersed into the constant temperature bath Based upon a study of equilibrium times, the solutions were allowed to equilibrate to the bath temperature of the study before removal of the sample corresponding to zero time Ampuls were removed at specified intervals and the reaction quenched by immersion into a bath containing a mixture of ice and water.

The samples were analyzed by means of the iron (III) hydroxamic acid colorimetric method Two ml. of the hydrolysis solution from the ampul was pipetted into a 10-ml volumetric flask containing 1.0 ml of 1 0 *M* hydroxylamine hydrochloride One ml of 2 0 *M* sodium hydroxide was added to each of the flasks and allowed to stand for fifteen minutes, acidified with 1 0 ml. of 2 0 *M* hydrochloric acid, mixed well, and the colored complex formed through the addition of 1 0 ml of 3 0 *M* ferric chloride solution in 0 1 *M* hydrochloric acid The mixtures in the flasks were again allowed to stand fifteen minutes, diluted to 10 0 ml with distilled water, and the absorbance determined against the blank at 530 mμ in a Beckman model DU spectrophotometer The extinction values for the sample were compared with values obtained for a standard solution containing 1.5 mg /ml. of ester

The analytical procedure required modifications when pH 6 buffer was used The hydrochloric acid solution was not of sufficient strength to lower the

TABLE I —VARIATION OF HYDROLYSIS RATES OF METHYL DL-α-PHENYL-2-PIPERIDYLACETATE WITH APPARENT HYDRONIUM AND HYDROXYL ION CONCENTRATION

Buffer Composition	Temp	Apparent pH[a]	Apparent $[OH^-] \times 10^{9b}$	k_1, min $^{-1}$	k^1, L mole^{-1} min^{-1}
0 0946 M HCl + 0 0054 M KCl	95	1 12		$5\ 82 \times 10^{-4}$	$7\ 68 \times 10^{-3}$
	80	1 10		$2\ 31 \times 10^{-4}$	$2\ 91 \times 10^{-3}$
0 0597 M HCl + 0 0432 M KCl	80	1 32		$1\ 23 \times 10^{-4}$	$2\ 57 \times 10^{-3}$
0 0119 M HCl + 0 0881 M KCl	95	2 02		$7\ 36 \times 10^{-5}$	$7\ 70 \times 10^{-3}$
	80	2 00		$3\ 17 \times 10^{-5}$	$3\ 17 \times 10^{-3}$
0 0330 M HCl + 0 050 M KHPhthalate	95	2 81		$3\ 78 \times 10^{-5}$	$2\ 44 \times 10^{-3}$
	80	2 76		$1\ 16 \times 10^{-5}$	$6\ 66 \times 10^{-3}$
0 0204 M HCl + 0 050 M KHPhthalate	95	3 22		$6\ 33 \times 10^{-5}$	
	80	3 16		$1\ 85 \times 10^{-5}$	
0 0040 M NaOH + 0 100 M KHPhthalate	80	4 16	3 70	$8\ 02 \times 10^{-5}$	$2\ 17 \times 10^{4}$
0 0473 M NaOH + 0 100 M KHPhthalate	80	5 17	37 9	$7\ 79 \times 10^{-4}$	$2\ 06 \times 10^{4}$
	65	5 11	16 2	$1\ 41 \times 10^{-4}$	$8\ 71 \times 10^{3}$
	50	5 07	6 45	$2\ 52 \times 10^{-5}$	$3\ 90 \times 10^{3}$
0 1816 M NaOH + 0 200 M KHPhthalate	80	6 13	345	$5\ 13 \times 10^{-3}$	$1\ 49 \times 10^{4}$
	65	6 11	162	$1\ 14 \times 10^{-3}$	$7\ 02 \times 10^{3}$
	50	6 07	64 5	$1\ 93 \times 10^{-4}$	$2\ 98 \times 10^{3}$

[a] pH values measured with glass calomel electrodes at the temperatures cited
[b] Calculated from apparent hydronium ion concentrations using pKw values 13 2603 at 50°, 12 9010 at 65°, and 12 5918 at 80°

pH for color development without the precipitation of ferric hydroxide When 1 0 ml of 3 0 M hydrochloric acid was used instead of 1 0 ml of 2 0 M acid, no precipitation resulted

RESULTS AND DISCUSSION

Hydronium Ion and Hydroxyl Ion Catalysis.—The kinetic information on the hydrolysis of methyl DL-α phenyl-2-piperidylacetate in solutions of unchanging hydronium ion concentration in the pH range 1 1–6 2 are summarized in Table I At constant hydronium ion concentration, pseudo first-order kinetics always were observed and Fig 4 illustrates typical semi-log plots of the fraction of unhydrolyzed ester as a function of time The first-order rate constants, k_1, were obtained from slopes of the regressions obtained by plotting 2 303 log (100/100 − x) against time in minutes, where 100 − x represents the percentage of unhydrolyzed ester at time t The estimated reproducibility of the first-order constants is within about 4% in the

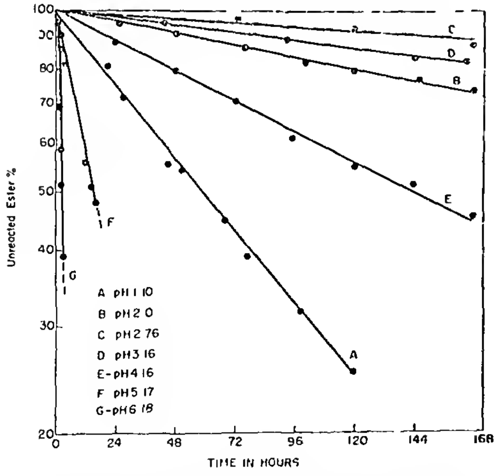


Fig 4 —Typical pseudo first-order rate plots for the hydrolysis of methyl DL α-phenyl-2-piperidylacetate at 80° at several pH levels

pH ranges 1–2 and 4–6, but only about 10% in the pH interval 2–4

Generally, the second-order rate constant for catalysis by a given species may be obtained from the ratio of the first-order rate constant to the thermodynamic activity of the species in the solution. However, most of the second-order rate constants cited in the literature for hydronium or hydroxyl ion catalysis were derived from the concentrations of hydronium or hydroxyl ions rather than the thermodynamic activities because of the analytical methods employed Inasmuch as the present study is concerned with the pH dependency of the hydrolysis and the rates were determined at a given hydronium ion activity, the value obtained from the ratio of the first-order rate constant to the hydronium ion activity will be referred to as the "apparent" second-order rate constant and designated as $k^1_{H^+}$

$$k^1_{H^+} = \frac{k_1}{A_{H_3O^+}}$$

In the pH range 1 1 to 2 0, the first-order rate constants appear to be a linear function of the hydrogen ion activity and the "apparent" second-order rate constant (Table II) was obtained from the slope of the line of a plot of −log k_1 *versus* the pH using the least squares method (Fig 5)

An added uncertainty is involved in the estimation of the second-order rate constant for the hydroxyl ion-catalyzed reaction since the hydroxyl

TABLE II —ESTIMATED APPARENT SECOND ORDER RATE CONSTANTS, ENERGY AND ENTROPY OF ACTIVATION FOR THE HYDRONIUM AND HYDROXYL ION-CATALYZED HYDROLYSIS OF METHYL DL-α-PHENYL-2-PIPERIDYLACETATE HYDROCHLORIDE

	H_3O^+	OH^-
$k^1 80°$, L mole^{-1} min $^{-1}$	$1\ 479 \times 10^{4}$	$2\ 871 \times 10^{-3}$
Ea	15,985 (±735)	12,350 (±210)
log A, sec $^{-1}$	5 79	10 15
$\Delta S^{\ddagger}$ 25°, cal / deg	−34 06	−14 04

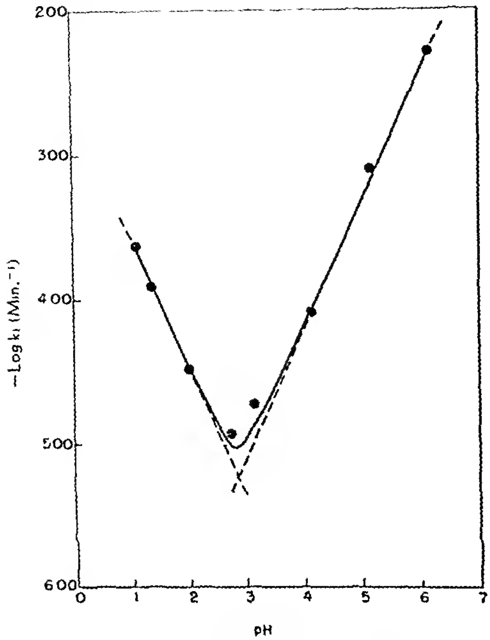


Fig. 5.—pH dependency of the hydrolysis of methyl DL-α-phenyl-2-piperidylacetate at 80°.

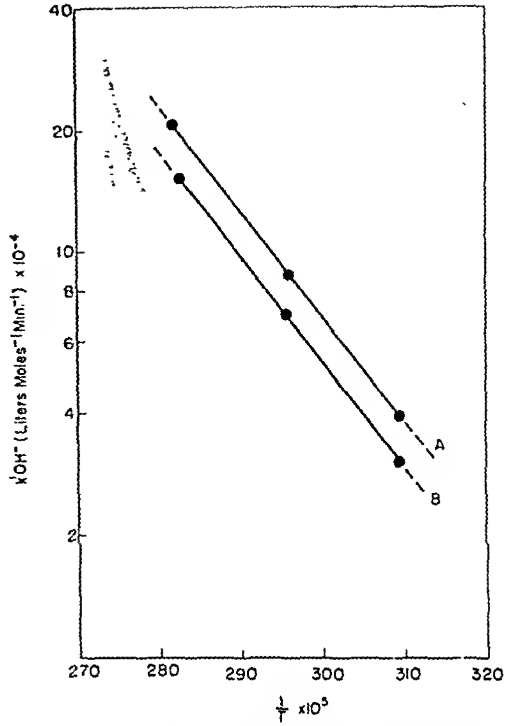


Fig. 6.—Arrhenius plot of the hydroxyl ion-catalyzed hydrolysis of methyl-DL-α-phenyl-2-piperidylacetate. A = pH 5.0, B = pH 6.0. Logarithm of the second-order rate constant against the reciprocal of the absolute temperature.

ion activity must be calculated from the measured pH. In addition, the "apparent" second-order rate constant, k'_{OH^-} calculated from the expression $k'_{OH^-} = k_1 A_{H_3O^+}/Kw$ is uncertain inasmuch as an accurate value of the ion product of water, Kw, at the temperatures above 60° and ionic strength of the hydrolysis mixture is not available. Inverse linear dependence of rate on the hydronium ion activity occurs in the pH interval 4–6 (Fig. 5). The value of k'_{OH^-} was calculated from the slope of the line and is cited in Table II.

As noted by Walker and Owens (19), second-order rate constants calculated from the hydroxyl ion activity are inaccurate to at least ±5% due to a corresponding uncertainty of the pH within ±0.02 units.

The limitations that we have applied to the kinetic constants presented in this paper undoubtedly apply as well to the velocity constants reported in the literature (20) and presented in Table III.

Temperature Dependency.—The activation parameters for the hydronium and hydroxyl ion catalysis are listed in Table II. Typical Arrhenius plots are shown in Fig. 6.

The second-order hydroxyl ion rate constants were calculated as $k_1 A_{H_3O^+}/Kw$ and as noted above are approximate as Kw is uncertain. The Kw values were calculated from the Harned and Owens (21) equation

$$\log Kw = \frac{6013.79}{T} - 23.6521 \log T + 64.7013$$

However, no correction in Kw was applied for the ionic strength of the solution.

Because of the uncertainty in the activation energies estimated from the second-order rate constants, the first-order rate constants were also used to determine Ea. The energy of activation, Ea, for the acid-catalyzed hydrolysis was found to be 15185 ± 725 cal. and the value for the base-catalyzed reaction was determined to be 25370 ± 570 cal. These values were obtained from the plots in Fig. 7. The activation energy obtained from the first-order rate constants of the hydroxyl ion-catalyzed reaction included the heat of ionization of water.

Absence of General Acid-Base Catalysis.—Bell (22) concluded from an examination of the data presented by Dawson (1) that the evidence for the general acid catalysis of ester hydrolysis is doubtful. The general acids present in the buffer systems used in this investigation are phthalic acid and the biphthalate ion (which is also a general base). To test the catalytic activity of phthalic acid, the hydrolytic rates at 95° were determined for solutions of the ester in 0.1 M HCl (0.0946 M HCl, 0.0054 M KCl) to which there had been added in different experiments 0.005 M, 0.01 M, 0.02 M, and 0.03 M phthalic acid. Within the experimental error, the second-order rate constants in these determinations were identical and corresponded to the rate found for hydronium ion catalysis. In another series of experiments utilizing the extraction method to follow the hydrolysis, the second-order rate constants at 95° were determined to be identical within the experimental error for solutions of the ester in 0.1 M HCl (0.0946 M HCl, 0.0054 M KCl) to which there had been added 0.05 M, 0.10 M, 0.20 M, 0.40 M, and 1.0 M acetic acid, respectively. Consequently, there is no evidence for catalysis by an undissociated acid.

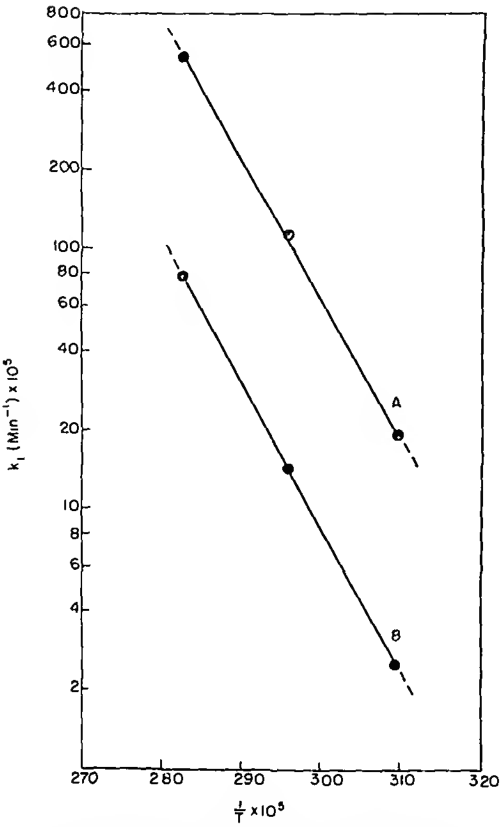


Fig 7—Arrhenius plot of the hydroxyl ion catalyzed hydrolysis A = pH 5 0, B = pH 6 0 Logarithm of the pseudo first-order rate constant against the reciprocal of the absolute temperature

Within the past year substantial evidence has accrued for general base catalysis in the hydrolysis of certain esters (7, 23–25) Inasmuch as the pKa$_2$ of phthalic acid is 5 51 appreciable amounts of the general bases biphthalate ion and phthalate ion are present in the buffers at pH 5 and 6 Hydrolysis of the ester (0 0056 M) in a buffer containing potassium biphthalate (0 0092 M) potassium sodium phthalate (0 0908 M) and sufficient potassium chloride to adjust the ionic strength to 0 8 afforded an initial second order velocity constant (followed only to 70% hydrolysis after which the pH of the solution increased) which was not changed when the buffer concentration was doubled at constant ionic strength Hence, it appears that the hydrolysis is catalyzed by neither biphthalate ion nor phthalate ion

The evidence presented that the hydrolysis is not a general acid base catalyzed process is tempered by the experimental error, and it is necessary to conclude that any contribution to the hydrolytic rate due to general acid base catalysis is negligible relative to the respective catalysis by hydronium ion and hydroxyl ion Thus, the hydrolysis appears to be specifically hydronium and hydroxyl ion catalyzed

Ionic Strength Effect—From the Bronsted Bjerrum equation (26) primary kinetic salt effects are expected for the specific hydronium ion and hydroxyl ion catalyzed hydrolysis of a cationic ester, a positive salt effect occurring in the hydronium ion catalyzed reaction and a negative salt effect in the hydroxyl ion catalyzed reaction The magnitude of the effect should be related to the proximity of the positive center to the site of attack by the catalytic species Characteristic primary salt effects have been observed in the acid and base catalyzed hydrolysis of carbethoxymethyltriethyl ammonium halide (11, 12, 27), and in the base catalyzed hydrolysis of methyl pyrrolidylacetyl salicylate hydrochloride (7) and acetylcholine (6) These effects do not appear to have been noted in the acid and base catalyzed hydrolysis of atropine (8 28) and acetylcholine (9) and in the base catalyzed hydrolysis of procaine (29)

Neglecting the minor effect of changing ionic strength in a solution of unchanging dielectric constant on the reaction between the attacking ion and the dipolar ester functional group the major ionic strength effect should conform to the expression applied to reaction between ionic species

$$\log k = \log k_0' + 1\ 02\ z_A z_B \sqrt{\mu}$$

where k_0' is the rate constant in an infinitely dilute solution z_A and z_B are the charges on the reactants A and B and μ is the ionic strength of the solution (26) This expression is applicable to very dilute solutions μ = 0 to 0 4 and marked deviations from the relationship have been observed with increasing ionic strength Nevertheless a plot of the logarithm of the second order velocity constants against the square root of the ionic strength should yield information regarding the primary kinetic salt effect

Figure 8 shows a plot of log $k^1{}_{H^+}$ as a function of $\sqrt{\mu}$ and illustrates the positive primary salt effect observed in the hydronium ion catalyzed reaction Hydrolysis of the ester (0 0093 M) in 0 1 M HCl and made up to final ionic strengths with sodium chloride was followed at 95° using the extraction method of analysis No correction of the ionic strength was applied due to expansion of the solvent Second order velocity constants were calculated from the first order constants using the measured hydrogen ion activities At μ = 0 50 $k^1{}_{H^+}$ = 9 80 × 10^{-3} L moles^{-1} min^{-1}, μ = 1 00 $k^1{}_{H^+}$ = 11 00 × 10^{-3} L moles^{-1} min^{-1}, and at μ = 2 00 $k^1{}_{H^+}$ = 13 08 × 10^{-3} L moles^{-1} min^{-1} Linear dependence of the plot of log $k^1{}_{H^+}$ against $\sqrt{\mu}$ is found however, the slope of the curve is 0 47 rather than the value of unity indicated in above equation The observed ionic strength effect for the acid catalyzed reaction appears to be relatively small Undoubtedly, the results obtained are only valid within the experimentally determined ionic

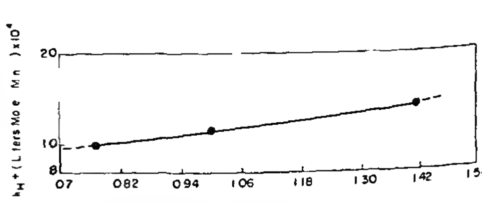


Fig 8—Influence of ionic strength on the velocity of hydronium ion catalyzed reaction

strength range and may not be extrapolated to lower ionic strengths where the slope is expected to increase

A comparable negative salt effect was observed for the hydroxyl ion catalyzed reaction. Employing the extraction method of analysis, first order velocity constants at 95° for the hydrolysis of the ester (0.0093 M) in buffers containing 0.2624 M NaH_2PO_4, 0.1504 M Na_2HPO_4 adjusted to final ionic strengths with sodium chloride were experimentally determined. Second order rate constants were calculated as previously described. At $\mu = 0.50$, $k'_{OH} = 3.54 \times 10^4$ L mole^{-1} min^{-1}, $\mu = 1.00$, $k'_{OH} = 3.30 \times 10^4$ L mole^{-1} min^{-1}, and at $\mu = 3.00$, $k'_{OH} = 2.77 \times 10^4$ L mole^{-1} min^{-1}. A plot of log k'_{OH} against $\sqrt{\mu}$ proved to be linear (Fig. 9) and illustrates a negative salt effect. As before, the slope of the plot, 0.307, deviates from unity. Because of the demonstrated primary salt effects, the rate constants determined in this study apply only to the vicinity of the experimental ionic strengths.

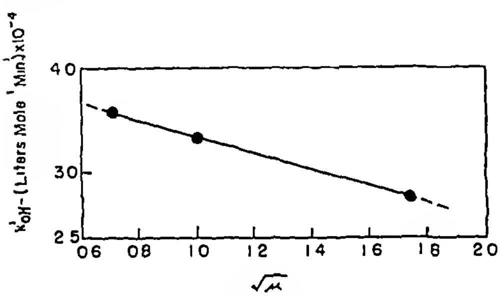


Fig. 9.—Influence of ionic strength on the velocity of the hydroxyl ion-catalyzed reaction.

The Hydrolytic Catenary.—The first order rate of the hydrolytic reaction in aqueous solution which is specifically catalyzed by the hydronium and hydroxyl ions is given by $k_1 = k_{H_2O}[H_2O] + k_{H^+}[H_3O^+] + k_{OH^-}(Kw)/[H_3O^+]$ (1). In the present paper k_{H^+} and k_{OH} are replaced by k'_{H^+} and k'_{OH}. When the rate of change of k_1 with a change in $[H_3O^+]$ is minimal, $dk_1/d[H_3O^+] = 0$ and $k'_{H^+} = Kw\, k'_{OH}/[H_3O^+]^2$, the hydronium ion concentration at this minimal overall velocity is noted as $[H_3O^+]_0$ and corresponds to the isocatalytic point since $k'_{H^+}[H_3O^+] = Kw\, k'_{OH}/[H_3O^+]_0$. The minimal velocity, v_0, at this isocatalytic point is given by $v_0 = 2k'_{H^+}[H_3O^+]_0$ or $v_0 = 2\sqrt{k'_{H_3O^+}\, k'_{OH^-}\, Kw}$ if the contribution of $k_{H_2O}[H_2O]$ is neglected.

The experimentally determined pH dependency of the hydrolysis at 80° is presented as a plot of $-\log k_1$ against pH in Fig. 5. The dominant feature of the curve is that the pH_0 appears to be 2.86 where the corresponding $k_1 = 9.33 \times 10^{-6}$. The pH_0 calculated from the second order velocity constants estimated from the slopes of the linear segments of the pH dependency curve, $k'_{H^+} = 2.87 \times 10^{-3}$ L mole^{-1} min^{-1} and $k'_{OH^-} = 1.48 \times 10^4$ L mole^{-1} min^{-1}, is 2.94 and the corresponding value of k_1 is calculated to be 6.59×10^{-6}. This low isocatalytic point is not unexpected qualitatively as a shift to a lower isocatalytic point parallels a large k'_{OH^-}/k'_{H^+} which in the case of a cationic ester is considerable for k'_{OH^-} is increased as a result of the electrostatic attraction of the molecule for the hydroxyl ion whereas k'_{H^+} is decreased through the electrostatic repulsion for the proton.

From the Arrhenius equations

$$k'_{H^+} = 3.68 \times 10^7 e^{-15,950/RT}$$

$$k'_{OH} = 8.57 \times 10^{11} e^{-17,350/RT}$$

the values of k'_{H^+} and k'_{OH^-} may be calculated for any given temperature. At 25°, for example, the calculated values, $k'_{H^+} = 7.02 \times 10^{-5}$ L mole^{-1} min^{-1} and $k'_{OH^-} = 7.59 \times 10^2$ L mole^{-1} min^{-1} yield the isocatalytic point 3.48.

The difference between the experimentally estimated velocity of the reaction at the isocatalytic point and that calculated from k'_{H^+} and k'_{OH^-} values may be attributed to the so called "spontaneous reaction" or $k_{H_2O}[H_2O]$. The contribution of $k_{H_2O}[H_2O]$ estimated in this way is 2.74×10^{-6} min^{-1} which is a substantially smaller value than the value 4.67×10^{-4} min^{-1} for $k_{H_2O}[H_2O]$ determined by Garrett (7) for the protonated ester methyl pyrrolidylacetylsalicylate hydrochloride. In addition, this exceedingly small difference between the estimated and calculated minimal velocities supports the postulate that there is no general acid base catalysis in the vicinity of the isocatalytic point.

For comparison purposes, the isocatalytic point of the hydrolysis of esters reported in the literature can be calculated from the specific velocity constants cited providing the ionic charge on the ester is the same in both determinations. Unfortunately, most second order constants for the hydroxyl ion-catalyzed reaction which have been determined apply to the nonprotonated ester. The isocatalytic points of many nonionic esters are in the range 5.3 to 5.6 (2). The value for ethyl aminoacetate is 3.8 at 20° (4) and hence it is apparent that the value of methyl DL-α-phenyl-2-piperidylacetate is of the proper order of magnitude. For comparison, the isocatalytic points of the cationic esters atropine and acetylcholine are 3.24 at 100° (8) and 3.73 at 25° (9), respectively. These values indicate that these compounds contain structural features which increase the ratio k'_{OH^-}/k'_{H^+}.

STRUCTURAL CONSIDERATIONS

The discussion presented above is consistent with Bender's mechanisms of ester hydrolysis (30) applied to a cationic ester. The recent studies of concurrent oxygen exchange and acid and alkaline hydrolysis of Bender (31) support the concept that a similar intermediate is involved in both the acid and base catalyzed reactions. Inasmuch as substituent groups will influence this intermediate, the effects of these groups may be compared for the hydronium ion and hydroxyl ion catalyzed reactions. Although methyl DL-α-phenyl-2-piperidylacetate may be viewed as a substituted methyl propionate or as an ester of heptanoic acid, it will be considered a substituted acetate for comparison purposes.

The low solubilities of nonionic esters in water has precluded kinetic studies of hydrolysis in this solvent, and it is necessary to extrapolate information from ethanol water and acetone water systems. In Table III, values of the energy of activation, Ea, for the hydronium ion catalyzed hydrolysis of methyl acetate are only very slightly higher in water than in 60% acetone and A increases almost

TABLE III.—ARRHENIUS CONSTANTS[a] FOR THE HYDRONIUM AND HYDROXYL ION CATALYZED HYDROLYSIS OF SOME ESTERS[b]

	Log A	Ea	Log A	Ea	Reference
Methyl DL-α-phenyl-2-piperidylacetate	5.79	15,980	10.16	12,350	([h])
Atropine[g]	5.41	17,200	10.32	12,700	(8)
Procaine[g]			8.87	12,000	(29)
Scopolamine methylbromide			8.61	11,800	(37)
Acetylcholine	7.67	16,570	9.00	12,180	(9)
Carbethoxymethyltriethylammonium chloride			10.82	12,700	(11)
Methyl acetate	7.64[c]	16,250	7.57[c]	11,500	(39)
	8.59	16,920			
Ethyl acetate			8.20[f]	14,200	(38)
			8.6[f]	14,700	(36)
	7.53[e]	16,200	5.9[e]	9,800	(33)
Ethyl propionate			6.78	10,720	(32)
			8.34[f]	14,500	(32)
Ethyl phenylacetate	7.08[d]	16,200			(33)
	6.97[e]	16,100	7.34[f]	14,100	(35)
Ethyl diethylacetate	8.08[e]	19,100	6.4	14,300	(33)
			7.96[f]	17,400	(40)
			7.8[f]	17,300	(36)

[a] $\log k = \log A - Ea/2.303RT$ as estimated from rate constants where k is $L.\ mole^{-1}\ sec.^{-1}$

[b] Solvent is water unless otherwise noted.

[c] 60% acetone.

[d] 85% ethanol.

[e] 70% acetone.

[f] 76.5% ethanol.

[g] Arrhenius parameters are for the protonated ester.

[h] This investigation.

by one factor of ten. It appears likely, therefore, that the Arrhenius parameters determined in 60 to 70% acetone for the acid catalysis are similar to those in water. However, considerable differences in the parameters have been observed for the base catalyzed reaction. The data of Tommila and Hietala (32) for the hydroxyl ion catalyzed hydrolysis of ethyl propionate in water and 76.5% ethanol (Table III) indicate that the energy of activation is approximately 3.5 Kcal./mole greater in the latter solvent and that A has increased by a factor of 15. Moreover, Davies and Evans (33) have compared values of Ea for alkaline hydrolysis in 70% acetone and 85% ethanol for the saponification of normal esters noting that the values are roughly 3.5 Kcal./mole greater in the hydroalcoholic solution. Therefore, while only gross comparisons are permitted, Ea values in water and acetone-water solutions are similar, whereas Ea values determined in 85% ethanol must be reduced by approximately 3.5 Kcal./mole for comparison purposes.

The effect of the α-phenyl substituent on acid and base catalyzed ester hydrolysis has been extensively investigated (34, 35). In the acid catalyzed hydrolysis of ethyl phenylacetate compared to ethyl acetate, Log A is reduced by 0.5 and Ea is unchanged. This effect has been attributed to the steric effect of the phenyl group, the negative inductive ($-I$) effect being negligible. The effect of the α-phenyl group on the base catalyzed reaction is to reduce Ea by 600 cal. and Log A by 1.3, these results being due to the $-I$ and steric effects. Effects of similar magnitude should be manifested by the α-phenyl group in methyl DL-α-phenyl-2-piperidylacetate. Thus the observed changes in the frequency factor cannot be significantly attributed to the α-phenyl group. Apparently, a large contribution to the changes in frequency factor is due to the piperidine substituent.

The union of the piperidine ring to the acetic acid residue is at the 2-position. The effect of an alkyl radical on the α-carbon of the acetic acid moiety should account for the effects other than that attributed to the nitrogen. In acid catalyzed hydrolysis, Ea is increased by 100 to 400 cal. as a result of the $+I$ effect and Log A is decreased by about 0.1 to 0.4 due to the steric effect (33). In base catalyzed hydrolysis, Ea may or may not be increased up to 400 cal. depending on the substituent ($+I$ effect) and in most cases a small decrease in Log A is observed, e. g., 0.4 to 0.8, which is very likely due to decreased hyperconjugation (36). An extreme case of the effects of α-alkyl substituents on ester saponification would be represented by ethyl diethylacetate which has an unusually high activation energy (Table III). These effects are relatively minor and consequently, the major contribution to the difference in the Arrhenius parameters of methyl DL-*α-phenyl-2-piperidylacetate* and acetic ester are due to the protonated nitrogen.

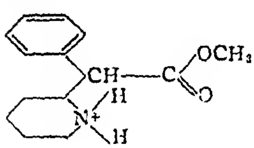
In Table III, the Arrhenius constants for hydronium ion catalyzed hydrolysis of methyl DL-α-phenyl-2-piperidylacetate, atropine, and acetylcholine are listed. The activation energies of these compounds are similar, whereas the nonexponential factor of atropine and methyl DL-α-phenyl-2-piperidylacetate are smaller than that of acetylcholine or a nonionic ester. Atropine is an ester of a secondary alcohol in addition to possessing a cationic center somewhat remote from the ester functional group and steric effects are manifested in both the alkyl and acid moieties. Thus a comparison of atropine and methyl DL-α-phenyl-2-piperidylacetate merely points up the large effect due to the cationic center in methyl DL-α-phenyl-2-piperidylacetate. It may be noted that in acetylcholine, without a contribution of steric hindrance, the hydrolysis rate is reduced by only a small extent as compared to a nonionic ester.

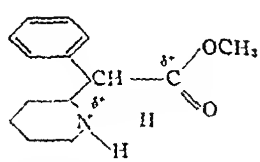
The activation energies for the hydroxyl ion catalyzed hydrolysis of the cationic esters listed in Table III are surprisingly similar to one another

considering their diverse structures. In both acetylcholine and carbethoxymethyltriethylammonium chloride, the quaternary nitrogen is closer to the center of hydronium ion attack than is the protonated nitrogen in methyl DL-α-phenyl-2-piperidylacetate with consequent greater negative inductive effects In addition, the Ea values of the cationic esters appear to be consistently larger than that of nonionic esters (values extrapolated to aqueous solution). As noted by Bell (11), the greater Ea is the result of an abnormally high collision factor resulting from the electrostatic attraction for the hydroxyl ion by the cationic ester. It appears that the collision factor makes a major contribution to the hydrolysis rate in cationic esters while the inductive effects are minor in importance

The frequency factors of the cationic esters are all greater than those of the nonionic esters and that of methyl DL-α-phenyl-2-piperidylacetate is seen to be similar in magnitude to those esters whose cationic center is closer to the carbonyl-carbon. It is evident that the large decrease in rate in the hydronium ion-catalyzed reaction and the increase in rate in the hydroxyl ion-catalyzed reaction of methyl DL-α-phenyl-2-piperidylacetate are accompanied by corresponding changes in the entropies rather than the energies of activation; at 25°, $\Delta S^{\ddagger}$ (20) corresponding to $k^1_{H^+}$ is −34.06 E.U. and to $k^1_{OH^-}$ is −14 04 E.U.

Conceivably, these results can be accounted for in terms of intramolecular hydrogen bonding of the protonated nitrogen to the carbonyl-oxygen.





Methyl-DL-α-phenyl-2-piperidylacetate

Inspection of a Fisher-Hirshfelder-Taylor model shows that such hydrogen bonding can readily occur. Placement of a fractional positive charge should enhance the approach of the hydroxyl ion to the carbonyl-carbon resulting in an increase in the collision factor and frequency factor. The approach of a proton to the methoxy-oxygen should be repelled by a carbonyl-carbon with enhanced electrophilic character, although the approach of the nucleophilic water molecule to the carbonyl-carbon should be facilitated Therefore, in the pH range 2 to 3, the hydronium ion concentration is sufficiently low that electrostatic repulsion of the available protons limits the reaction velocity, but as the hydrogen ion concentration is increased, the attraction of the carbonyl-carbon for the nucleophilic water molecules results in an increased reaction velocity Alternately, increased velocity with increasing hydronium ion concentration may be due to a loss of stability of the hydrogen bond as the proton approaches the carbonyl-oxygen

CONCLUSIONS

1. The hydrolytic reaction appears to be catalyzed specifically by hydronium and hydroxyl ions.

2. A positive primary salt effect seems to be present for the hydronium ion-catalyzed reaction and a negative salt effect has been observed for the hydroxyl ion-catalyzed reaction.

3. The pH of maximum stability for the ester in aqueous solutions is 2.86 at 80°.

4. At a pH of 2 or less and 4 or higher, the reaction is essentially catalyzed by hydronium and hydroxyl ion, respectively.

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Veratrum Alkaloids XXXII*

The Structures of Germitetrine and Some Related Hypotensive Ester Alkaloids

By S. MORRIS KUPCHAN and C. IAN AYRES

The structures of germitetrine and several related hypotensive esters of germine have been elucidated. The chemically-related series germitetrine, desacetylgermitetrine, and neogermbudine are formulated as I, IV, and V, respectively.

ALKALOIDAL MIXTURES obtained from *Veratrum album* are employed in the treatment of hypertension. One of the most powerful hypotensive constituents of these alkaloidal mixtures is germitetrine (1–4) whose structure elucidation was recently described (5) in a brief preliminary communication. The present report details the evidence for assignment of structure I to germitetrine.

Alkaline hydrolysis of germitetrine has afforded the known alkamine germine (II) (6), two mole equivalents of acetic acid, one mole equivalent of (*l*)-2-methylbutyric acid, and one mole equivalent of the low melting isomer of 2,3-dihydroxy-2-methylbutyric acid (2, 4). Methanolysis of germitetrine was shown to yield two degradation products. The loss of one acetate grouping led to the triester desacetylgermitetrine, whereas removal of both acetate groupings resulted in conversion to the naturally-occurring germine diester, neogermbudine (4). The acetate grouping methanolyzed in the conversion of germitetrine to desacetylgermitetrine has been provisionally assigned to C_7 on the basis of molecular rotation differences and the characteristically facile methanolysis of C_7 acetates (7).

In a recent review (8), the four acyl groups of germitetrine were tentatively assigned to positions C_3, C_7, C_{15}, and C_{16} of germine. We were led to question this proposal on the basis of a preliminary structure-activity generalization which appears to exist among the hypotensive veratrum derivatives. To date, all the hypotensive naturally-occurring germine esters of known structure possess a free hydroxyl group at C_{16} (7).

An indication that the C_3, C_7, C_{15}, C_{16} tetraester structure was untenable came from the observation that germitetrine was completely stable toward sodium periodate. The proposed tetraester would have a free glycol present in its DMB residue and would therefore be readily attacked by sodium periodate. Furthermore, germitetrine was shown to possess a free C_{16} hydroxyl group in the following manner. Chromic acid titration showed the presence of only one secondary hydroxyl group. The oxidation product, germitetrone (VI) (m. p. 222–223° decompn.), on alkaline hydrolysis gave the diosphenol (VII) previously obtained from neogermitrone and 16-dehydrogermine 3,6,7,15-tetraacetate (6, 7). Thus germitetrine is a germine 3,7,15-triester in which the secondary hydroxyl group present in the DMB residue is acylated, thus accounting for the four mole equivalents of acid produced on hydrolysis.

The location of germitetrine's more easily methanolyzed acetyl residue at C_7 requires that desacetylgermitetrine be a germine 3,15-diester in which the secondary hydroxyl group of the DMB residue is acylated. The remaining structural questions were settled by studies on neogermbudine, the germine mono-MB mono-DMB which is obtained upon methanolysis of desacetylgermitetrine (see Table I). Neogermbudine consumed 1 mole equivalent of sodium periodate in agreement with assignment of the acyl residues at C_3 and C_{15}. The specific location of each substituted butyryl residue was determined by use of the reported selectivity of the sodium borohydride reduction of esters of hydroxyacids (9, 10). When neogermbudine was treated with sodium borohydride, the dihydroxybutyryl ester grouping was selectively removed and a known (11) germine mono-(*l*)-2-methylbutyrate was isolated. This compound consumed 1.1 mole equivalents of sodium periodate in agreement with the germine 15-(*l*)-2-methylbutyrate structure tentatively assigned on other grounds (7). Furthermore, the amorphous oxidation product showed infrared absorption at 3.65, 5.62, and 5.80 μ characteristic of the aldehydo-γ-lactone resulting from cleavage of the ring A glycol (6). Thus, neogermbudine is germine 3-DMB-15-MB (V), desacetylgermitetrine is germine 3-HMAB 15-MB (IV), and germitetrine is germine 3-HMAB 7-Ac 15-MB (I).

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Part XXXI in the series: S. M. Kupchan, N. S. Johnson, and S. Rajagopalan, *Tetrahedron*, in press.

CHART 1

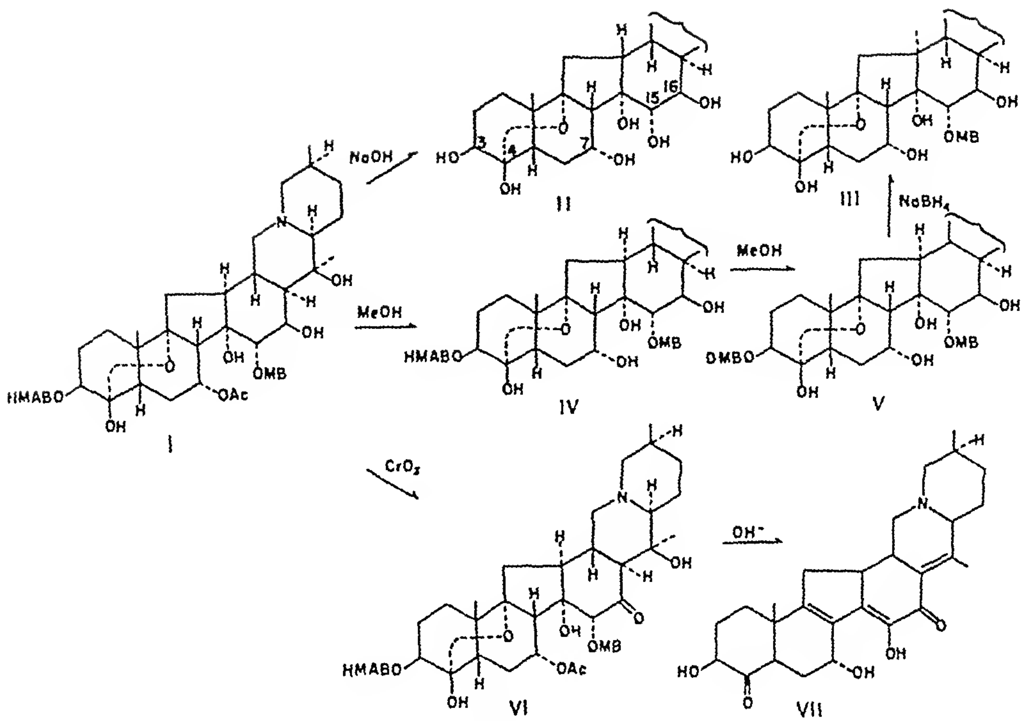

TABLE I

Germitetrine (germine-mono Ac-mono MB-mono HMAB)	MeOH[4,7] ⟶	Desacetylgermitetrine (germine-mono MB-mono HMAB)	MeOH[4] ⟶	Neogermbudine (germine-mono MB-mono DMB)
↓ NaOH				↓ $NaBH_4$
Germine				Germine-15-(*l*)-2-methylbutyrate

MB = (*l*) 2 methylbutyryl DMB = (*l*) 2,3 dihydroxy 2 methylbutyryl HMAB = 2-hydroxy 2 methyl 3 acetoxybutyryl

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree Ultraviolet absorption spectra were determined on a model 11 MS Cary recording spectrophotometer and 95% ethanol was used as solvent Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as a solvent Microanalyses were carried out by Dr S M Nagy and his associates at the Massachusetts Institute of Technology on samples dried at reduced pressure at 110°

Oxidation of Germitetrine to Germitetrone (VI) — Germitetrine (39 3 mg), m p 229–231° (decompn), in glacial acetic acid (5 cc) was treated at room temperature with a solution of chromic anhydride in acetic acid containing 0 2% of water (5 cc , 0 06 *N*) Aliquot parts were titrated at intervals With the aid of the appropriate blank, the rate of consumption of chromic acid was shown to be as follows: forty minutes, 0 74 of the theoretical amount of reagent required for the oxidation of one secondary hydroxyl group, one hour, 0 80 of theoretical, two hours, 0 89 of theoretical, twenty hours, 1 16 of theoretical

Germitetrine (300 mg.), m p 229–231° (decompn) in glacial acetic acid (4 cc) was treated with 0 66 *N* chromic anhydride in 98 5% acetic acid (8 cc) The dark brown mixture was allowed to stand at room temperature for seventy-five minutes The reaction mixture was cooled in an ice bath and treated first with dilute sodium bisulfite to a green color and then with dilute ammonia until alkaline The solution was extracted with chloroform and the chloroform solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness at reduced pressure. The residue crystallized from 95% ethanol in the form of colorless prisms (250 mg), m p 220–221° (decompn) Recrystallization of a sample for analysis from 95% ethanol afforded colorless prisms, m p 222–223° (decompn), $[\alpha]_D^{22}$ −167° (c 1 08, py)

Anal —Calcd for $C_{41}H_{61}O_{14}N \cdot H_2O$: C, 60 79, H, 7.84 Found: C, 61 18; H, 7 88

In a volatile acid determination (12) 26 00 mg of the ketone yielded an amount of acid equivalent to 13 53 cc. of 0 007317 *N* sodium thiosulfate, calcd for two mole equivalents of acetic acid and one mole equivalent of (*l*)-2-methylbutyric acid, 13 16 cc

Alkaline Treatment of Germitetrone.—Germitetrone (30 mg), m p 220–221° (decompn), was

treated with methanol (3 cc) and 50% sodium hydroxide solution (0 1 cc) and the solution was heated under reflux for twelve minutes The resulting red solution was treated dropwise with glacial acetic acid until a yellow solution was obtained, and then evaporated at reduced pressure The residue was treated with water (5 cc), brought to pH 9 with dilute ammonia, and extracted with chloroform The chloroform solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness at reduced pressure The residue was dissolved in ether (5 cc) and the solvent was allowed to evaporate at room temperature The light brown powdery residue (12 mg) showed m p 170–180° (decompn), λ_{max} 320 mμ (ϵ 13,000), 285 mμ (ϵ 7,000), $\lambda_{max}^{0\ 01\ N\ KOH}$ 372 mμ (ϵ 10,000), 345 mμ (ϵ 8,000)

Conversion of Neogermbudine to Germine 15-(*l*)-2-methylbutyrate (III)—Neogermbudine[1] (100 mg , Ayerst McKenna and Harrison Ltd R7593, m p 149–152°) in methanol (3 cc) was treated with a solution of sodium borohydride (100 mg) in methanol (9 cc) The solution was allowed to stand at room temperature for fifteen hours, acidified with glacial acetic acid, and evaporated at reduced pressure at 30° The residue was dissolved in water, cooled in an ice bath, made alkaline with dilute ammonia, and extracted with chloroform The chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated at reduced pressure to dryness The residue (72 mg) was chromatographed on Merck acid washed alumina (10 Gm) and 40 cc fractions of eluate were collected Fractions 1, 2, and 3 eluted with chloroform, fractions 4 and 5 eluted with 1% methanol in chloroform, and fractions 6 and 7 eluted with 2% methanol in chloroform did not yield any solid material Fraction 8, eluted with 7% methanol in chloroform, yielded a resin consisting mainly of starting material, as indicated by paper chromatographic comparison with neogermbudine The procedure employed was that described by Levine and Fischbach (13) for "slower moving" alkaloids Fraction 9, eluted with 10% methanol in chloroform, yielded 17 mg of residue, fraction 10, eluted with the same solvent, gave 10 mg of residue, fraction 11, eluted with 15% methanol in chloroform, gave 8 mg of residue (total solid eluted 61 mg) Fractions 9,10, and 11 consisted largely of germine 15 (*l*) 2 methylbutyrate, as indicated by paper chromatographic comparison with an authentic sample[2] The three fractions were, therefore, combined in chloroform and filtered through a column of Merck acid washed alumina (1 Gm) to remove some colored impurities Evaporation of the filtrate gave a colorless residue (35 mg) which was crystallized from chloroform ether to give elongated prisms (9 mg), m p 225–229° (decompn) with slight preliminary browning A second crop of prisms (15 mg), m p 216–219° (decompn), was also obtained The melting point of the first crop material was not depressed by admixture with the authentic sample of germine 15 (*l*) 2 methylbutyrate The infrared spectra of the respective samples were identical

[1] We thank Dr G S Myers of Ayerst McKenna and Harrison Ltd for a generous gift of neogermbudine

[2] We thank Dr J Bolger of Riker Laboratories Inc for a comparison sample of germine 15 (*l*) 2 methylbutyrate

Sodium Periodate Oxidations—The titrations were performed as in our earlier studies (6) Germitetrine consumed no sodium periodate in nine hours Neogermbudine consumed 0 84 mole equivalent in two hours, 0 95 in six hours, and 1 03 in twenty four hours

Germine 15 (*l*) 2 methylbutyrate (27 3 mg), m p 225–229° (decompn), was dissolved in 5% acetic acid (2 cc) and water (3 cc) and 0 08 *M* sodium periodate solution (5 cc) were added Titration of a 2 cc aliquot after two and one half hours showed an uptake of 1 07 mole equivalents Titration of a second 2 cc aliquot after twenty hours showed an uptake of 1 30 mole equivalents The remainder of the solution (6 cc) was made alkaline with dilute ammonia and extracted with chloroform Evaporation of the chloroform left an amorphous residue which showed infrared absorption at 3 65, 5 62, and 5 80 μ These bands have been shown to characterize the Ring A aldehydo γ lactone structure (6)

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Intermediary Metabolism of Ergot I*

Amino Acid Metabolism

By GUNNAR GJERSTAD

A comparative analysis of the amino acid metabolism of parasitic and saprophytic ergot is reported. *Identification was effected by two-dimensional paper partition chromatography.*

In RECENT YEARS considerable interest has centered around the problem of making the ascomycete, *Claviceps purpurea* (Fries) Tul., produce the medicinally important ergot alkaloids when grown in synthetic or semisynthetic substrates (1). Occasional reports that alkaloids have been extracted from the fungus cultivated in artificial media have proved to be nonreproducible in the United States using local isolates of *Claviceps* (2).

In a previous publication (3) the influence of the addition of several chemical compounds to synthetic substrates was investigated in an attempt to test for any precursor effects. The results indicated strongly that the failure of alkaloid formation was most plausibly not due to a nutritional deficiency of the employed substrates.

Since alkaloids were not formed, it appeared of great interest to investigate if *Claviceps* would show any other metabolic differences when grown on rye as compared with the same strain raised in submerged cultures.

EXPERIMENTAL

Cultures.—The initial isolate of the employed fungus was obtained through courtesy of Eli Lilly & Co., Indianapolis, and described as rye ergot of a very high alkaloid yielding strain. The culture which has been maintained for several years on Kirchhoff's agar medium (4), was inoculated on Balbo rye Tennessee by dipping the heads into a conidio spore suspension, and in the course of fifty days, mature sclerotia developed. The same conidio culture was grown in submerged culture in the laboratory for an identical period of time.

Media.—Since we were interested in analyzing the resulting mycelia for amino acids, it was considered important not to add organically bound nitrogen to the substrates. The employed medium was patterned according to the one described by Stoll, *et al.* (5), in their successful work with ergot isolated from *Pennisetum typhoideum* Rich., and consisted of the following:

Mannitol	50.0 Gm.
Sucrose CP	20.0 Gm.
Potassium dihydrogen phosphate	1.0 Gm.
Magnesium sulfate $7H_2O$	0.3 Gm.
Manganese sulfate	0.10 Gm.
Ferrous sulfate $7H_2O$	0.004 Gm.
Succinic Acid	5.4 Gm.
Distilled water to make	1,000 cc.

* Received November 10, 1958, from the College of Pharmacy, University of Texas, Austin.

This medium, which was slightly acidic, was subsequently adjusted to a pH of 5.6, by use of approximately 4 ml. of stronger ammonia solution per 1,000 ml. Thus, NH_3 was the only nitrogen source of the fungus. The produced fungal material was processed as described in an earlier publication (3).

Analytical.—The sclerotia and the saprophytic fungal material were subjected to the same analytical procedure following in principle that of Grøger and Mothes (6): 1.000 Gm. of dried substance was extracted exhaustively with petroleum benzin to remove all lipid material. The marc was subsequently extracted with 20 ml. of 50% ethanol overnight in a horizontal shaking machine. The resulting filtered extract was evaporated to dryness in a Virtis freeze drier. The isolated soluble material was then dissolved in 0.5 ml. of a mixture of ethanol and water (4 + 2 by volume) to which was added one drop of chloroform as a preservative. The resulting solution was subjected to paper partition chromatography, ascending formation being employed. Several solvent systems recommended in the literature (7) as satisfactory for amino acid analyses were tried, but with the majority, relatively poor separation was obtained. The most satisfactory solvent system was found to be in the first direction azeotropic aqueous *n*-propanol (71.7% w/w). In the second direction, a mixture of 80% phenol in water was employed. The chromatograms were sprayed routinely, after drying, with ninhydrin solution (7), and all identified spots were substantiated by cochromatography with authenticated amino acids.

RESULTS AND DISCUSSION

In the saprophytic cultures polymorphism was less pronounced than in the previously employed media (3). Growth was relatively slow, as evidenced by a lag phase of seven days. The fungal mat was brownish blue in color, and a microscopic inspection revealed an abundant formation of conidia in all five parallel experiments. A test for sucrose in the culture filtrate was negative; the pH had decreased to 5.0, indicating a slight acidification during cultivation. The yield of dried fungal material was more uniform than experienced in previous work (3) and ranged from 0.913 to 1.183 Gm. per 70 ml. of medium.

The lipid production showed a significant difference in the two ergot samples. The sclerotia contained 17.6% of petroleum ether soluble substance as against 12.3% in the saprophytically developed fungal material. This finding may be due to the difference in cultivation temperature. The sclerotia were developed on free land during the hot Texas

summer, while the artificial cultivation took place under constant temperature in an air-conditioned laboratory

The results of the amino acid study are illustrated in Figs 1 and 2 Several ubiquitously occurring amino acids were identified, however, no appreciable qualitative difference was found between the artificially and the parasitically developed ergots Spots 9 and 13 are unidentified and are probably polypeptides

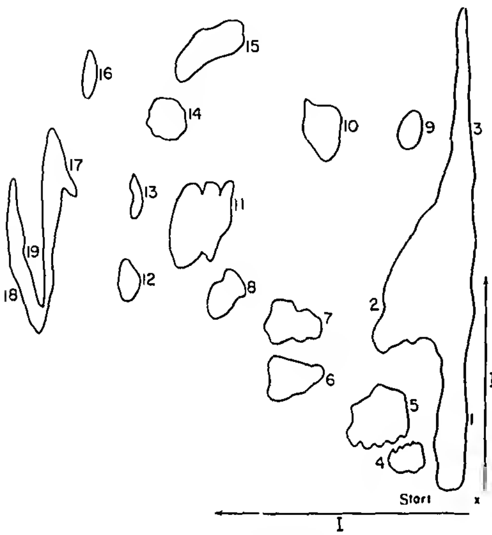

Fig 1 —Free amino acids in sclerotia

I, Azeotropic *n*-propanol *II*, Phenol-water (80 + 20) *1*, Lysine *2*, Arginine *3*, Histidine *4*, Aspartic acid *5*, Glutamic acid *6*, Serine *7*, Glycine *8*, Threonine *9*, Unidentified. *10*, α-Aminobutyric acid *11*, Alanine *12*, Tyrosine *13*, Unidentified *14*, γ-Aminobutyric acid *15*, Valine *16*, Proline *17*, Isoleucine *18*, Phenylalanine *19*, Leucine

Quantitatively, there seemed to be a marked difference with respect to the metabolism of alanine and glutamic acid as judged by the size of the test spots Hyphae produced the greatest quantity of alanine, while sclerotia on the other hand were richer in glutamic acid

Spots 1, 2, and 3 were identified by cochromatography with known pure amino acids, however, it is highly possible that these diffuse spots also represent various proteins which react with ninhydrin. A study of the latter is presently under way.

The most striking result of our investigation is that we were unable to ascertain in any culture the presence of tryptophan, which in many publications has been suggested as the precursor of all ergot alkaloids (3). Its detection was attempted with several specific chromatogram developers for tryptophan, as listed in current literature (7) This finding is in agreement with that of Grøger and Mothes (6). and also in line with the result of

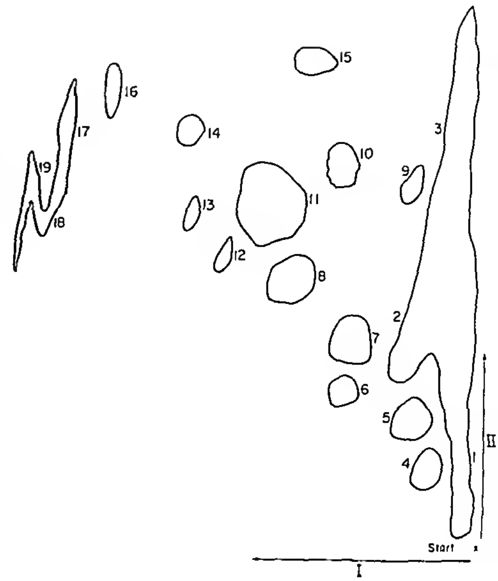

Fig 2 —Free amino acids in saprophytic fungal material

I, Azeotropic *n*-propanol *II*, Phenol-water (80 + 20) *1*, Lysine *2*, Arginine *3*, Histidine *4*, Aspartic acid *5*, Glutamic acid *6*, Serine *7*, Glycine *8*, Threonine *9*, Unidentified *10*, α-Aminobutyric acid *11*, Alanine *12*, Tyrosine *13*, Unidentified *14*, γ-Aminobutyric acid *15*, Valine *16*, Proline *17*, Isoleucine *18*, Phenylalanine *19*, Leucine

Suhadolnik and Loo, who, according to a recent publication (8) on ergot failed to obtain radioactive alkaloids *in vivo* by use of tagged tryptophan as precursor Thus, it may seem justified to conclude that tryptophan is not directly involved in the biosynthesis of lysergic acid or the ergot alkaloids The absence of sulfur containing amino acids is also noteworthy, although no speculations as to its significance will be offered at this time.

The abundant occurrence in both types of fungal material of the aminobutyric acids indicates that these hardly are involved in the alkaloid biosynthesis Since no organically bound nitrogen was present in the saprophytic media, a strong anabolitic capacity of *Claviceps* for N is in evidence

Studies of the comparative metabolism of carbohydrates, organic acids, lipids, and polypeptides of ergot are in progress.

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Ion Exchange Separation Method for Microdetermination of Tropane Alkaloids in the Presence of Morphine*

By EERO SJÖSTRÖM† and ANTTI RANDELL

A method has been developed for the quantitative determination of micro amounts of atropine and scopolamine in the presence of large quantities of morphine. The components are first separated on an anion exchange column and the tropane alkaloids then colorimetrically determined. It is found that when solutions of relatively high methanol concentrations are used the tropane alkaloids are not hydrolyzed by the strongly basic resin. By contrast, in aqueous solution, a considerable saponification takes place and the results obtained are consequently too low. The method has been applied to the determination of tropane alkaloids in injection solutions. The method is simple and gives very satisfactory accuracy and reproducibility.

SEVERAL ANALYTICAL ion exchange methods have been reported during recent years for isolation of morphine and other phenolic alkaloids from different materials (1-7) as well as for separation of morphine from nonphenolic alkaloids (8-12). The latter methods are based on the fact that morphine is quantitatively retained in a column filled with a strongly basic resin, whereas the nonphenolic alkaloids pass through. Since the alkaloid salts are converted into the corresponding free bases, they can be titrated in the effluent. According to this principle codeine and atropine, for instance, can be determined in the presence of morphine.

Although the ion exchange-titration methods can be used in many cases, their applicability is limited to relatively high concentrations. Yet, in medical preparations the tropane alkaloids are often present as a microcomponent. In these cases the titration becomes very inaccurate. Furthermore, the inaccuracy is seriously increased with increasing ratios of morphine/atropine (or scopolamine) for the following reasons: (*a*) "pure" morphine of pharmacopeial quality usually contains up to 0.5% of nonphenolic alkaloids which are titrated with the tropane alkaloids, (*b*) ionized substances such as the neutral salts, are also exchanged and titrated with the tropane alkaloids, and (*c*) it is difficult to avoid traces of alkaline impurities dissolved from the resin even when working carefully under controlled conditions.

The object of the present investigation was to develop a method for quantitative microdetermination of tropane alkaloids in the presence of large amounts of morphine. For the reasons given above, it was evident that the determination after the separation must be based on some more sensitive and specific procedure than titration. The methods commonly used for this purpose are colorimetric and are based on nitration of the tropic acid portion of the tropane alkaloid molecule. Accordingly, in using these methods it is essential that no saponification should take place in the resin, otherwise the tropic acid liberated is retained in the column. Experiments showed that when the alkaloids are passed through the column in aqueous solution a considerable saponification occurs. On the other hand, it was found that if methanol or ethanol solutions are used, no saponification takes place and the alkaloids pass quantitatively into the effluent. Since morphine is completely retained by the resin even from these solutions, it was possible to devise a method suitable for the microdetermination of tropane alkaloids in the presence of morphine.

EXPERIMENTAL

Equipment and Materials.—The experiments were performed with the commercial resin Dowex 1 with various degree of cross-linking, having round particles of 50 to 100 mesh. The ion exchange columns were of standard type, measuring 6 x 110 mm. The resin was converted into the hydroxyl ion form with about 50 ml. of 1 *N* sodium hydroxide and washed with water to neutral against methyl red. Before the separation experiments the water was displaced from the columns with the solvent (about 50 ml.) and gas bubbles, if they appeared, were removed (17).

The morphine used was of pharmacopeial quality (Ph. F. VII) and purified in the following way. About 300 mg. of morphine hydrochloride was dissolved in 100 ml. methanol and passed through the ion exchange column. The column was afterwards washed with absolute methanol until the nonphenolic impurities had completely passed into the effluent (about 50 ml.). The completeness of the washing was checked by means of ultraviolet measurement, i. e., the column was washed until no absorption could be observed in the ultraviolet. The

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morphine taken up was eluted with 0 33 M boric acid solution in absolute methanol. After evaporation of the boric acid and the methanol from the eluate, the remaining free morphine was dissolved in ethanol and neutralized with hydrochloric acid. The resulting morphine hydrochloride was recrystallized from ethanol and dried in a vacuum desiccator in the presence of sulfuric acid

Atropine sulfate and scopolamine hydrobromide of pharmacopeial quality (Ph F VII) were used as such The methanol and all the reagents were from Merck (Darmstadt). The ethanol from Oy Alkoholiliike Ab for spectrophotometric purposes was used

The measurements were carried out with a Zeiss spectrophotometer, model PMQ II, 1-cm cells being used

The Analytical Procedure.—After numerous experiments a suitable procedure for the estimation of atropine and scopolamine was devised by modifying the method described by Bratton and Marshall (13, 14) This method is based on the reduction of the nitrated alkaloid and its subsequent diazotization The color produced is stable The resulting procedure is carried out as follows: Transfer an aliquot of the ion exchange effluent containing about 0 06 to 0 14 mg alkaloid into a 50-ml beaker and evaporate to dryness on a water bath Allow to cool and add fuming nitric acid (0 6 ml) and evaporate the excess of nitric acid on a water bath. Dissolve the residue in 50% ethanol (10 ml) Add 10% hydrochloric acid (5 ml) and 0 3 Gm zinc dust Cover the beaker with a watch glass and allow to stand for ten minutes on a water bath Cool the solution to room temperature and filter it into a 25-ml volumetric flask by washing the residue of zinc with water (2 × 3 ml) Add 1% sodium nitrite solution (1 ml), mix, and let stand for ten minutes. Add 2 5% ammonium sulfamate solution (1 ml), shake, and let stand for ten minutes Finally, add 1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution (1 ml), made up to volume with water, and measure after about thirty minutes at 550 mμ Prepare a blank with the reagents and standards, simultaneously with the sample At the concentration range mentioned the corresponding absorbance is about 0.3 to 0 7 Since the reproducibility of the results seems to be good, the alkaloid content can even be calculated from a standard curve

In connection with the saponification experiments atropine was also determined in the ultraviolet region, its maximum being at 211 mμ. For the detection of morphine, ultraviolet measurements were performed at its maximum of 286 mμ

RESULTS AND DISCUSSION

Uptake of Morphine Hydrochloride.—The break-through curve of the purified morphine hydrochloride in 80% methanol is shown in Fig 1 As can be seen, the break-through at a flow-rate of 1 ml /min. is relatively sharp even when a normally cross-linked resin Dowex 1X-8 is used. Morphine could not be detected by ultraviolet measurement in the effluent of either of the resins when 70 ml of 0 5% morphine hydrochloride solution was passed through. Accordingly, within the limits of the measurement sensitivity, not more than 0.02% has passed through the column. Both resins were able

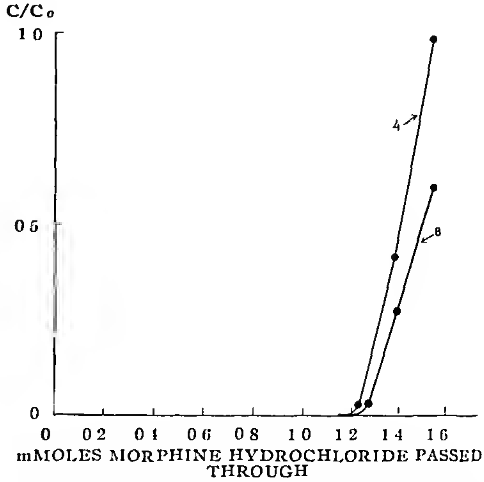

Fig. 1.—Break-through curve for morphine hydrochloride (0.0155 mole/1 in 80% (w/w) methanol). Curve 4: Dowex 1X-4. Curve 8: Dowex 1X-8 Total capacities of the columns 3.45 and 3.36 meq.

to take up about 1.2 mM (375 mg.) of anhydrous morphine hydrochloride quantitatively, i e , about 70% of their theoretical total capacity for morphine hydrochloride

It must be pointed out that when morphine hydrochloride of pharmacopeial quality (Ph. F VII) was passed through the column, the effluent showed a strong absorption in the ultraviolet The impurities were identified by paper chromatography. The main impurity, about 0 4% of the original morphine preparation, was proved to be codeine.

Hydrolysis of Tropane Alkaloids by the Resin.—The influence of solvent composition on the saponification of the alkaloids was studied by using methanol and ethanol solutions with various amounts of water

The experiments were performed by passing 20 ml (2 mg) of the alkaloid salt solution through the column, which was afterwards washed with solvent to obtain an effluent volume of 50 ml. The results are summarized in Table I As can be seen from these data, hydrolysis is quite naturally favored by

TABLE I —HYDROLYSIS OF ATROPINE AND SCOPOLAMINE IN METHANOL-WATER AND ETHANOL-WATER MIXTURES[a]

Alcohol Concentration, (by Weight) %	Found in Effluent, % — Atropine[b] in Methanol-Water	Atropine[b] in Ethanol-Water	Scopolamine[c] in Methanol-Water
100	100 0		97 0
90	98 1	102 0	95 5
80	100 0		93.5
70	98 1	96 5	93 5
60	97 8		86 5
50	96 5	92 2	79 5
40	89 7		66 5
30	79 7	69 5	59.5
20	60 4		50 0
10	53 5	21 8	.
0	33 0		.

[a] Resin Dowex 1X-4, Flow-Rate 1 ml /min [b] Ultraviolet determination [c] Colorimetric determination.

the increasing water content. Within the limits of experimental error all the atropine passes through the column if the methanol content is 70% or more On the other hand, in aqueous solution, 67% of the atropine is saponified. It can be noted, too, that scopolamine is more easily saponified than atropine. However, it must be pointed out that the results are not strictly comparable, since the flow-rate could not be kept absolutely constant Nevertheless, it can be inferred from the experimental data, that for the quantitative recovery of scopolamine the methanol concentration must be kept near 100%

These results are in good agreement with earlier observations concerning the ester interchange of estradiol benzoate by strongly basic resin. It was namely found that in absolute methanol and ethanol only transesterification takes place and no hydrolysis (15) The reason is that the hydroxyl ions are completely displaced by the alkoxide ions according to the following reaction:

$$MeOH + HO^- \rightleftarrows MeO^- + H_2O$$

The apparent equilibrium constant for this reaction was found to be 11 1 (16). This means that if the methanol concentration in the resin phase is 70%, 97% of the hydroxyl ions of the resin are converted into methoxide ions The colorimetric method used did not reveal whether the tropane alkaloids are interchanged in the column, since the methyl tropinate possibly formed is coestimated A paper chromatogram of the effluent indicated, however, that within the limits of the accuracy, i e , about ±10%, no ester interchange takes place

Separation of Tropane Alkaloids From Morphine.—In order to check the effectiveness of the separation method, alkaloid mixtures varying in the ratio of morphine/atropine (or scopolamine) were passed through the column The experiments were performed by passing 20 ml of the alkaloid salt mixture through the column, which was afterwards washed with solvent to obtain an effluent volume of 50 ml The results obtained (Tables II and III) may be considered satisfactory even though the deviations become greater when the tropane alkaloid content is extremely low compared with the morphine present The errors probably arise from the impurities dissolved from the resin and from the nonphenolic impurities of the morphine

Elution of Morphine.—The column can be regenerated with a 0 33 M boric acid solution in absolute methanol This solution was earlier observed to be an excellent eluting agent for estradiol (17, 18). The results obtained in the present investigation show that boric acid in methanol is also very effective for displacing morphine This observation is of importance as difficulty has earlier been reported in this process (19, 20) Apart from its elution efficiency, the methanolic boric acid has the added advantage that it evaporates readily. The elution curves for different resins with various degree of cross-linkage are illustrated in Fig 2 It can be noted that morphine is, quite naturally, more easily displaced from the resins with a low degree of cross-linkage However, a good recovery is also achieved by using normally cross-linked resins It is interesting to note that by substituting methanol for ethanol, the elution efficiency is markedly decreased, as can be seen from Figs 3 and 4

Application of the Method to Medical Preparations.—The method has been used with success in this laboratory for a period of half a year At the beginning, the Vitali reaction was used for the determination of tropane alkaloids However, when it was found that the reproducibility was often poor, the method was changed to that described in this paper The ion exchange separation combined with this method has proved to be of very satisfactory accuracy and reproducibility. Some typical data are collected in table IV.

It may be emphasized that glycerol, which is often

TABLE II —SEPARATION OF ATROPINE FROM MORPHINE[a]

Added: Morphine Hydrochloride, mg	Added: Atropine Sulfate, µg	Found in Effluent, Atropine Calculated as Sulfate, µg	Error, %
50	1,000	945	−5 5
50	500	500	±0 0
50	200	197	−1 5
100	200	204	+2 0
100	100	116	+16 0

[a] Dowex 1X-4, 80% (w/w) methanol, Flow-Rate 1 ml /min

TABLE III —SEPARATION OF SCOPOLAMINE FROM MORPHINE[a]

Added: Morphine Hydrochloride, mg.	Added: Scopolamine Hydrobromide, µg	Found in Effluent, Scopolamine Calculated as Hydrobromide, µg.	Error, %
50	1,000	975	−2 5
50	500	510	+2 0
50	200	209	+4 5
100	200	216	+8 0
100	100	106	+6 0

[a] Dowex 1X-4, Absolute Methanol, Flow-Rate 1 ml./min

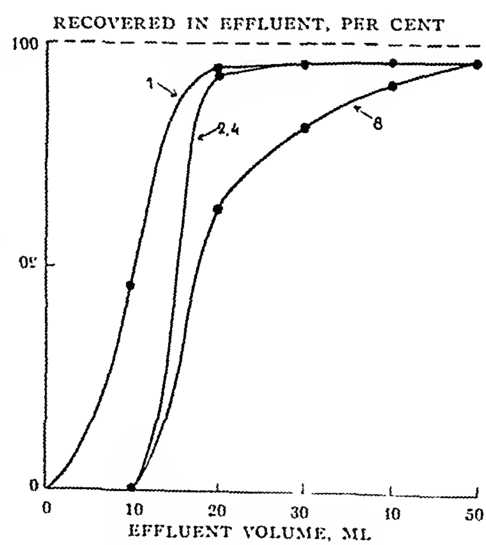

Fig. 2.—Elution of morphine with 0.33 M boric acid in absolute methanol. The numbers of the curves indicate the cross-linking of the corresponding resin.

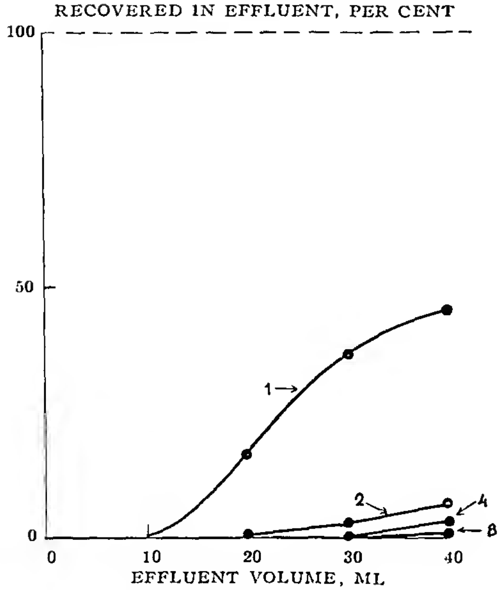

Fig 3 —Elution of morphine with 0 33 *M* boric acid in absolute ethanol The numbers of the curves indicate the cross-linking of the corresponding resin

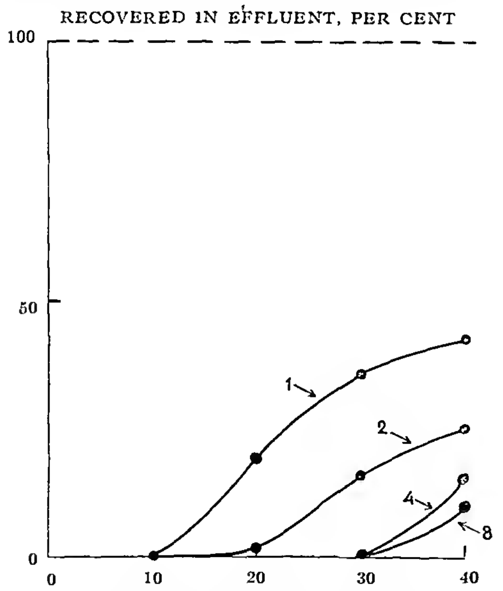

Fig. 4 —Elution of morphine with 0 33 *M* boric acid in 70% (w/w) ethanol. The numbers of the curves indicate the cross-linking of the corresponding resin.

present in injection solutions of this type, interferes with the colorimetric estimation. Therefore, it must be separated from the mixture If an anion exchange column alone is used, glycerol passes into the effluent along with the tropane alkaloids This problem has been solved by coupling a cation exchange column in series with the anion exchange column Thus, the morphine is first removed by the anion exchanger, the tropane alkaloids passing with

TABLE IV.—DETERMINATION OF ATROPINE AND SCOPOLAMINE IN INJECTIONS

Composition and Content, mg		Atropine or Scopolamine Found Calculated as Respective Salts mg	Error, %
Morphine hydrochloride	20	0 250	±0 0
Atropine sulfate	0 25	0 275	+10 0
Glycerol	50	0 250	±0 0
Alcohol	120	0 260	+4 0
Distilled water ad	1 ml.		
Morphine hydrochloride	20	0 580	−3 3
Scopolamine hydrobromide	0 6	0 561	−6 5
Glycerol	50	0 580	−3 3
Alcohol	120	0 580	−3 3
Distilled water ad	1 ml		

the glycerol into the cation exchange column Glycerol can easily be washed from the column with the solvent The tropane alkaloids taken up by the cation exchanger are finally eluted with a methanol solution containing ammonia and determined in the eluate The details of the resulting procedure are as follows Pipet a suitable amount of the injection solution (2–4 ml) into a beaker and add absolute methanol (100–150 ml) Transfer this solution through an anion exchanger column and let the effluent pass directly into a cation exchange column of the same size (Dowex 50X–4, H^+-form, 50/100 mesh) Wash the anion exchange column with absolute methanol (about 50 ml) and also let the washing methanol pass through the cation exchanger Elute the tropane alkaloids from the cation exchanger with a solution which is prepared by diluting 60 ml strong ammonia solution to 100 ml with methanol, and let the eluate pass directly into a 100-ml volumetric flask. Take from this solution an aliquot (10 ml), evaporate the solvent on a water bath, and determine the alkaloid content as described

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An Examination of the Oil of the Seeds of Persimmon (*Diospyros Virginiana* L., Fam. *Ebenaceae*)*

By CECIL P. CLOUGHLY and HENRY M. BURLAGE

A fixed oil was extracted by petroleum ether from the seeds of *Diospyros Virginiana* L., representing approximately three per cent of the weight of the seeds. The oil is semidrying, and its properties are similar to sesame oil. The petroleum ether extract has a dark, reddish brown color, musk-like odor, and a bland taste like olive oil. It consists of 80.24 per cent unsaturated fatty acids, of nearly equal parts of oleic and linoleic acids. The saturated fatty acids consist of stearic and palmitic acids, with traces of myristic and arachidic acids. Saturated acids amounted to 16.25 per cent of the total weight of the oil. The amount of unsaponifiable matter and the physical constants were determined. A qualitative identification of the saturated fatty acids was made by chromatography using the technique developed by Holasek and Winsauer. Separation of the saturated and unsaturated fatty acids was effected by Pelikan and Von Mikusch modification of the Bertram oxidation method. Stearic and palmitic acids were identified after their separation by fractional crystallization from methanol at low temperatures. Results indicate that: The chemical constituents of the oil of the seeds of *Diospyros Virginiana* L. are similar to sesame oil; yield of the oil from the seeds is 3.41 per cent; and possibility exists that this oil might be useful in the formulation of perfumes or cosmetics where the oil might be used to advantage.

ALTHOUGH the persimmon tree is a familiar part of the countryside in the South, it has never been extensively cultivated in this country It is considered by some persons to be an undesirable plant, and studies have been made to destroy the tree by poisons when it is discovered growing voluntarily (1)

DeSoto, in 1539, learned from the Indians the value of persimmon fruit as a supplement to his diet, and he remembered this in the narrative of his expedition written eighteen years later at Evora in 1557 (2)

The common name, persimmon, is of Virginia Indian origin, and its longer, botanical name is *Diospyros Virginiana* L There are approximately 160 varieties of *Diospyros*, mostly Asiatic The common American species ranges southward from southern Pennsylvania to the Gulf Coast and westward to Texas It is also found in southern New York, West Virginia, Ohio, Indiana, Illinois, southern Iowa, Missouri, and Kansas In Florida the tree is sometimes called "possum wood" because the fruit attracts the small mammal

The tree produces what is technically a berry, and is described as being subglobose, varying to depressed or elongate, 3 to 4 cm in diameter The fruit has one to eight seeds that are flat, elliptic, and very hard The main characteristic of the fruit is that it is exceedingly astringent when green, but sweet and edible when ripe or after exposure to frost

* Received April 9, 1959 from the University of Texas College of Pharmacy Austin

Abstracted from a thesis submitted to the Graduate School of the University of Texas by Cecil Pershing Cloughly in partial fulfillment of the requirements for the degree of Master of Science

Honorable mention, Lunsford Richardson Pharmacy Awards 1959

The leaves of the tree look something like the American magnolia tree, and are four to six inches long, thick, and oval The leaves in summer are a shiny dark green, but during the autumn they become brilliant orange or scarlet, adding much to the beauty of the countryside

The bark of the tree is nearly black and is divided into squares The tree grows to an average height of 50 feet, although there are some exceptions as noted in Illinois and Indiana of persimmon trees growing as tall as 100 feet. It spreads from roots and often covers abandoned fields and roads with a scrubby growth. It also grows well from seeds and is propagated by migratory birds The wood is very hard and dense, and after the tree has matured a hundred years, the wood is known as "heartwood" This is used in making valuable hardwood objects As previously noted, the fruit of this tree has been cultivated in Asia since ancient times, and many varieties of persimmons have been developed (3).

EXPERIMENTAL

Preliminary Treatment of Seeds.—Seeds for the study were taken from the forests of trees of *Diospyros Virginiana* L, growing in Orange County, North Carolina The meaty material of the fruit that clung tenaciously to the seeds was removed by rubbing vigorously between hard-finished pieces of cloth The seeds were reduced to a moderately coarse powder in a Wiley grinding mill and then treated with a series of selective solvents in succession in a continuous extraction apparatus in the following order: petroleum ether, absolute ether, chloroform, U S P, absolute alcohol, and 70% ethyl alcohol

The research was confined to the petroleum ether extract since examination of the other extracts showed that they contained materials removed

from the seed body but no oil After the petroleum ether was removed under controlled conditions, a preliminary examination of the resultant substance showed that the dark reddish-brown oil (3 41%) was soluble in the usual organic solvents and was readily saponified Elemental analysis showed negative tests for nitrogen, sulfur, halogens, and phosphorus.

Persimmon seed oil is classified with the semidrying oils (4) The Helmer and Mitchell hexabromide test (5) gave negative tests for linolenic acid Figure 1 shows the percentage increase in weight of persimmon seed oil and laboratory samples of linseed and sesame oil when exposed to the atmosphere under controlled conditions Chemical properties of the oil obtained by following standard methods (6, 7, 8) are shown in Table I

The infrared spectrum of persimmon seed oil showed no unusual infrared absorption bands produced by the oil at particular wavelengths

Partition Chromatography of Saturated Fatty Acids from Persimmon Seed Oil.—The soap solution remaining after saponification of a weighed sample of the oil was dissolved in hot distilled water and treated with diluted sulfuric acid until neutral to phenol red The water-insoluble fatty acids were separated from the mixture by extraction with petroleum ether An aliquot portion of this extract was measured, the solvent evaporated off, and the residue of fatty acids then accurately weighed The concentration of the fatty acids in the petroleum ether extract was next adjusted so that 0 35 Gm of such acids were present in each 25 ml of solution, and a method was sought to identify the acids by use of paper chromatography This concentration was chosen because it would furnish approximately 66 μg of acids in each drop of solution to be used in the procedure

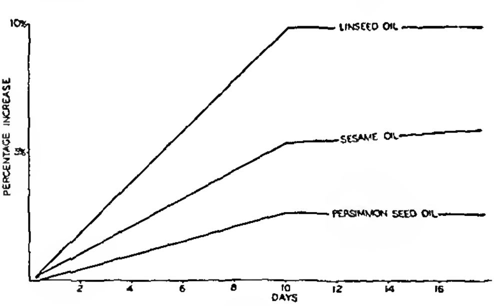

Figure 1.

TABLE I —PROPERTIES OF PERSIMMON SEED OIL

Elemental Analysis	
Nitrogen	Absent
Sulfur	Absent
Halogens .	Absent
Phosphorus	Absent
Refractive Index (25°) .	1 4800–1 4801
Acid value	30 3
Ester value. . .	129 3
Iodine value	104 01
Saponification value	160 23
Thiocyanogen number	78 04
Unsaponifiable matter	4 9%

Oxygen absorption—Gain in weight of 2.8/100 parts in ten days.

The solution of fatty acids was transferred by a lambda pipet to strips of paper impregnated with 0 5% $KAl(SO_4)_2.12H_2O$. These strips were then developed in standard chromatography jars according to the method of Holasek and Winsauer (9) The developer used consisted of approximately 300 ml of a solution of freshly distilled carbon tetrachloride, freshly distilled methyl alcohol, and concentrated ammonia (81:18:1). After developing for twelve to fourteen hours, the strips were removed, dried in an oven at 60° for twenty minutes, and then sprayed with a 0 1% solution of Rhodamin B in 0 05 N hydrochloric acid. The papers were again dried in the oven and examined under ultraviolet light Separated fatty acids showed up as dark red spots in a red to violet background Persimmon seed oil fatty acids developed spots on such chromatograms with R_f's of 70, 80, and indefinite spots at R_f's of 65 and 90 This indicated that palmitic and stearic acids were present, and possibly myristic and arachidic acids

Separation of Saturated from Unsaturated Fatty Acids.—Separation of the saturated from the unsaturated fatty acids was accomplished by the Pelikan and Von Mikusch modification of the Bertram oxidation method (10) Using this method, a sample of persimmon seed oil weighing 5 281 Gm yielded 0 9048 Gm of saturated fatty acids, and represented 17 1% of the total weight of the oil Since the isolated fatty acids often carry over small portions of unsaturated fatty acid, it was necessary to perform a correction to the first calculation of saturated fatty acids The iodine value of the isolated fatty acids was computed by the U. S P method (11) and was found to be 4.6

The following formula was used to calculate the corrected value for the saturated fatty acid content of the oil:

$$G = 100/S \quad W = IW/90$$

where G = corrected saturated fatty acids content in %, W = weight of the isolated fatty acids (0 9048 Gm), I = iodine value of the isolated fatty acids (4 6), and S = weight of the sample used (0 9048).

By substitution in the above equation, it was determined that the correct percentage of saturated fatty acids in persimmon seed oil amounted to 16 26 % (10) See Table II

TABLE II —FATTY ACID COMPOSITION OF PERSIMMON SEED OIL

Saturated fatty acids present: arachidic (trace), myristic (trace), stearic and palmitic.	16 26%
Unsaturated fatty acids ·	
Linoleic acid (calculated as glycerides)	39 36%
Oleic acid (calculated as glycerides)	40 88%

Determination of Unsaturated Fatty Acids.—The thiocyanogen and iodine values, both previously determined, were used to calculate the percentages of hypothetically pure glycerides in persimmon seed oil (12) By using these values and a formula recommended by the Association of Official Agriculture Chemists (13) it was determined that persimmon seed oil consisted of the following unsaturated fatty acids: linoleic acid, 39.36% and oleic acid, 40.88%.

Separation of the Saturated Fatty Acids.—The saturated fatty acids remaining from the permanganate oxidation experiment were separated from each other quantitatively by fractional crystallization from methanol at low temperatures (14) Palmitic and stearic acids were identified in the fractions by mixed melting points.

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Air-Suspension Technique of Coating Drug Particles*

A Preliminary Report

By DALE E. WURSTER

A new method of rapidly coating drug particles of widely varying size and shape is presented. In this process, the drug particles are coated and dried while suspended in an upwardly moving current of air. Solutions and suspensions of coating materials in both water and volatile organic solvents are employed. The drying of the coated particles is accomplished at either room or elevated temperatures, depending on the solvent used. Some fundamentals and an introduction to practical applications of the process are given.

CONSIDERABLE EFFORT has been expended to develop more rapid and more precise methods of coating compressed tablets and other drug particles. Progress in this field includes refinements of the conventional pan coating method and compression coating. The process described in this paper is a departure from both of the previous methods. It is simple, extremely rapid, and appears to be adaptable for large volume coating operations.

APPARATUS

The apparatus consists of a vertical column which is constricted at the bottom and expanded at the top (Fig 1) The air velocity in the constricted portion of the column is so great that particles entering this region are immediately propelled upwards In the expanded portion of the column the air velocity is greatly decreased This decreased velocity will not support the particles and they fall to the central or working region of the column In Fig 1 it can be observed that the column also contains a side vent Both the side vent and constricted portion of the column contain abjustable plates with which the velocity of the air stream in the working region of the column can be controlled. Various modifications in the design of the column can be employed; however, the upper portion is usually expanded to decrease the

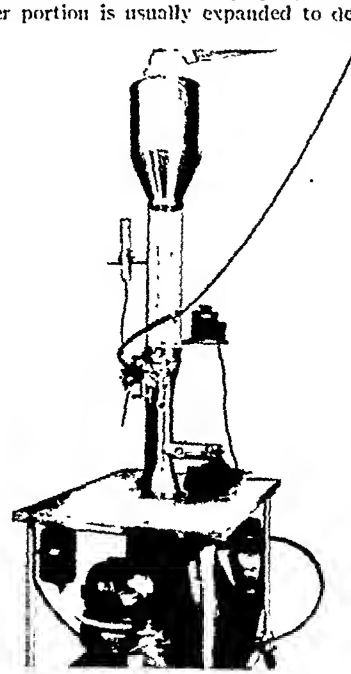

Fig. 1.—Air-suspension coating apparatus,

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velocity and prevent the loss of particles Various segments of the column may also be selected as the coating region but the velocity must be controlled to confine the particles to this region A constant speed blower (Buffalo Forge Co Type 5E) positioned at the base of the column serves as the air source A thermostatically controlled gas burner is utilized to heat the air prior to its entrance to the blower An atomizer for spraying the coating solution is normally positioned immediately below the working region of the column, but its position can be varied for specific uses

PROCESS

The process consists simply of supporting particles in a vertical column with an upwardly moving air stream during which time the coating solution is atomized onto the suspended particles The velocity of the air stream is adjusted so that the suspended particles are maintained in a relatively confined working region of the column The drying time of the coat can be governed by controlling either the atomization rate, the temperature of the supporting air stream, or both

A simple method for approximating the velocity required to suspend spherical particles in air was found to be useful in this work Since the particles are suspended in an air stream rather than just agitated on a supporting surface but still are not transported, the particles were compared on the basis of their weight and cross sectional area to equivalent inches of water (static pressure) The velocity required to support the particle was therefore calculated as follows

$$V = K\sqrt{M/(A/k)}$$

where V is the velocity in feet per minute, K the velocity required to maintain a static pressure of one inch of water (4,005 ft /min) M is the weight of the particle (Gm) A is the cross sectional area (cm ^{2}), and k is the weight of a column of water having the dimensions 1 cm x 1 cm at the base and 2 54 cm high (2 54 Gm)

It is apparent that particles can be held in a suspended state in an upwardly moving current of air for an indefinite time period if the velocity required to support the particles is held constant It is also apparent that if particles in the column are uniformly exposed to the coating solution and the atomization rate of the solution held constant, a linear relationship between the gain in weight of the particle and time of atomization should be obtained This, of course, assumes that possible attritional effects are not significant The thickness of the coat on the particle will not, of course, increase in a linear manner since the specific weight of the coating solids obtained in a given atomization time period must cover an ever increasing surface area A linear relationship between the cube of the radius of the particle or the increase in volume and time should, however, be obtained

EXPERIMENTAL

Air Velocity. Since the vertical column contains adjustable plates to control the air stream, the column was first calibrated in order to determine the average velocity obtained in the column for each setting of the plates The average column velocities were determined with a pitot tube and an Ellison (2 inch) inclined draft gauge

Large hollow spheres were employed to study the average velocity required to suspend spherical particles The density of the particles was increased by injecting a given quantity of water into them The particles were then accurately weighed In this manner the density of the particles was increased but the effective area upon which the upward moving air impinged was held constant The average velocity required to suspend the sphere was then obtained from the column calibration curve

Studies on Coat Build-Up.—Small, uniform, spherical sugar particles were initially coated with "Carbowax" 6000 in order to increase the particle size sufficiently so that they could be easily measured with a micrometer caliper The average particle size was determined and a known weight of these particles was placed in the coating column The particles were coated with a solution containing *25% "Carbowax" 6000 (w/v) in methyl alcohol* The atomization rate was maintained at approximately 56 34 ml per minute (2,000 ml atomized in thirty-five and one half minutes) Due to the volatility of the solvent the column was operated at room temperature At the end of the above time interval the contents of the column were removed, spread out, and allowed to dry at room temperature (approx twenty four hours) until a constant weight for the batch was obtained

The average weight of the coated particles obtained in each test was determined from five samples, each containing 150 particles

The average particle diameter was obtained by measuring the individual particles in the above 150 particle samples with a micrometer caliper

Coating Procedure.—The velocity required to suspend compressed tablets and the smaller particles was first calculated in order to obtain the approximate column setting and was then determined experimentally Two to four kilos of the particles to be coated were introduced into the column and atomization of the coating solution started as soon as the particles reached the operating height in the column In the case of compressed tablets the lower half of the Lucite tube (see Fig 1) was the working height, whereas with smaller particles the entire length of the tube was employed The particles were immediately removed from the column after the desired volume of coating solution had been applied

When aqueous solutions were employed the column was operated at a temperature of 49–50° and the coating solution was maintained at 85–90° With volatile solvents the column was operated at room temperature

Solutions employed for applying rounding coats to compressed tablets included simple syrup simple syrup, 67% and 33% of a "Carbowax" 4000 solution (50% w/v), simple syrup, 50% and 50% of a "Carbowax" 4000 solution (50% w/v), and 56 5% simple syrup, 19 8% starch paste (4 25% w/v), 22 6% "Carbowax" 4000 (50% w/v), and 1 1% Tween 20 These syrups were atomized at a rate of 47 to 48 ml per minute Two liters were thus applied in approximately forty two minutes

Suspensions of insoluble powders in the syrups were also successfully applied.

Solutions containing 25% wax in carbon tetrachloride were atomized at rates of 50 to 90 ml. per minute on 16–20 and 30–35 mesh particles. Solutions containing 20 or 30% of a lipoprotein or a resin in isopropanol were atomized at rates of 10 to 20 ml. per minute on 40–80 mesh particles.

DISCUSSION AND DATA

Air Velocities.—The experimental velocities required to suspend large spheres of varying density were compared with the velocities calculated according to the given equation. According to the equation a linear relationship should exist between the square root of M/A and the velocity. In this respect a plot of the experimental findings is in fair agreement with a plot of the calculated values (Fig. 2). It is emphasized here, however, that regions of both high and low velocities exist in this particular column. The experimental values given here are thus average velocities.

A single spherical particle in the column was maintained at a uniform height; however, during the actual coating process the suspended particles rise in the high and fall in the low velocity regions of the column. Nevertheless, the velocity requirements for the process were approximated according to the described method.

Coat Build-Up.—In Fig. 3 the average weight of the particle is plotted against the time and indicates that a straight line relationship between the weight of the particle and the time of atomization can be obtained with the process. In Fig. 4 the cube of the radius is plotted against time and again shows a straight line relationship. This tends to indicate that the adherence of the coating solids and the coat build-up occur in a uniform manner.

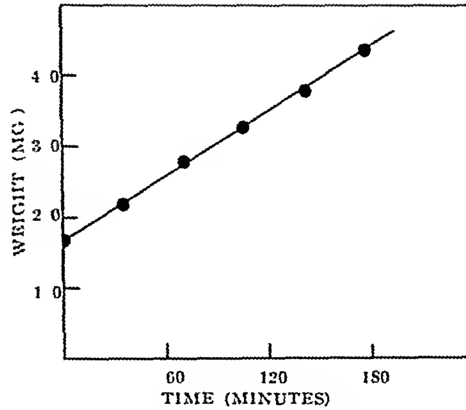

Fig. 3.—Average weight of particles *vs.* time.

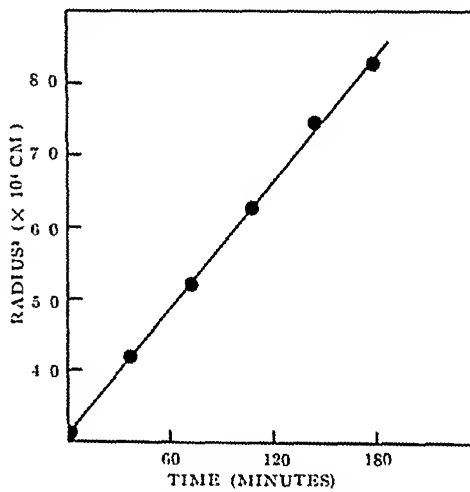

Fig. 4.—Average particle size *vs.* time.

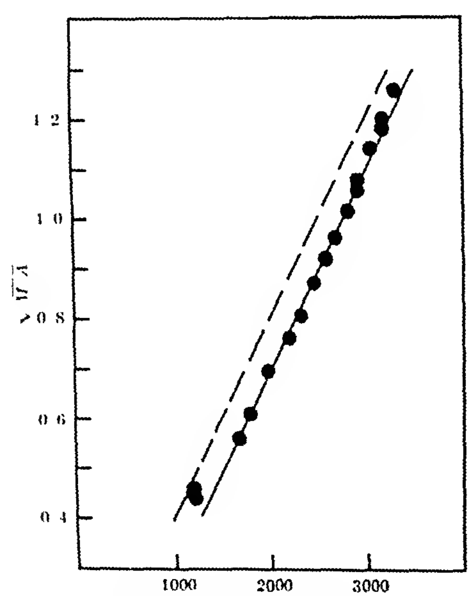

Fig 2.—Supporting velocity for spheres of varying density. (– – —Calculated.——Experimental.)

Loss of Solids.—Only a relatively small amount of the atomized solids was lost in the operation of the column when the small sugar particles were coated. This, no doubt, is due to the fact that the extremely large number of particles in the column makes the escape pathway of the atomized particle a very tortuous one. Thus, it is almost impossible for the atomized particle to traverse the length of the column without impinging upon the surface of the particles to be coated. Only a small amount of the atomized material collected on the wall of the column In Table I it can be observed that less than 1% of the starting material is lost when the loss is calculated on the basis of the total solids introduced into the column. When the loss is based solely on the atomized solids the loss is considerably larger (approx. 3–7%). This latter value, however, appears to be erroneous since the column was not originally designed for small particles and some of particles as well as the atomized solids were lost in the column joints. This is particularly obvious in the last three tests in the table where only one-half the weight of atomized solids was introduced into the column and the per cent loss is approximately two times that of the previous tests. This tends to indicate that the amount of particles lost in the column joints was

TABLE I —LOSS OF MATERIAL IN COLUMN

Test No	Weight of Particles Introduced into Column, Gm	Weight of Solids in Atomized Solution Gm	Total Solids Introduced into Column, Gm	Total Solids Recovered from Column, Gm	Loss of Total Solids, %	Loss if Based on Atomized Solids, %
1	1,500 00	500	2,000 00	1,983 02	0 85	3 39
2	1,982 66	500	2,482 66	2,470 10	0 51	2 51
3	2,469 68	500	2,969 68	2,950 40	0 66	3 85
4]	1,475 20	250	1,725 20	1,709 00	0 94	6 48
5	1,708 42	250	1,958 42	1,938 90	1 00	7 81
6	1,938 24	250	2,188 24	2,172 20	0 74	6 41

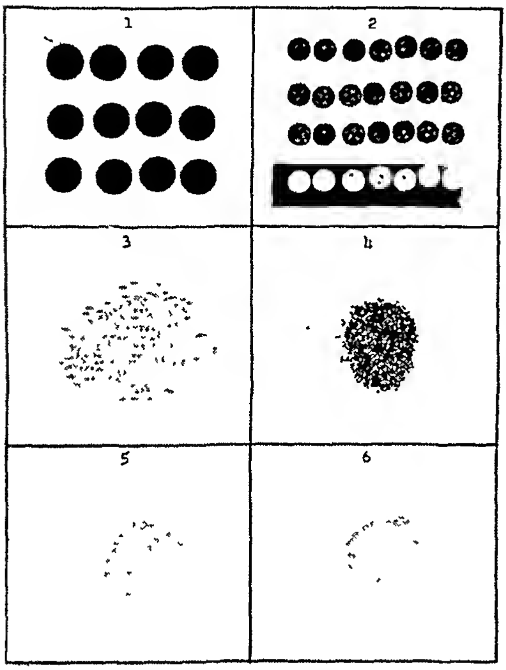

Fig 5 —Coated particles of various sizes. 1, rounding coat on compressed tablet, 2, compressed tablet made from granulation prepared by coating procedure; 3, wax-coated particles (16–20 mesh), 4, wax-coated particles (35–40 mesh), 5, lipoprotein-coated particles (40–80 mesh), 6, resin-coated particles (40-80 mesh)

the same as in the previous tests and distorts the true value of the atomized solids lost

Practical Applications.—This paper includes only exploratory work in the realm of practical applications Subsequent papers will deal more specifically with this phase of the work However, the process seems to be well suited for applying rounding coats to compressed tablets and for coating smaller particles with a variety of materials

The rounding coats on tablets were applied in a remarkably short time (twenty-five to forty-five minutes). Simple syrup yielded a highly crystalline and somewhat rough coat. however syrups which formed a more amorphous coat gave a smoother product It is believed that refinements of the atomization system and formulation changes will make it possible to apply the finishing coats to tablets by this method

Since a wide variety of substances can be applied by this method small particles can be coated for many purposes such as to change the rate of drug release from the primary particle, to inhibit the chemical decomposition of drugs, and to modify flow properties Other exploratory work indicates that film type coats on compressed tablets and tablet granulations can be prepared by this method Also, materials such as those used in compression coating can be applied to drug granules and tablets prepared with a single compression operation with an ordinary tablet machine

Figure 5 shows particles of several sizes which were prepared by this process

SUMMARY

A new air-suspension method for the rapid application of coating materials to small particles and compressed tablets is described.

The described process does not appear to be limited by either the coating materials or the solvent system, as both polar and nonpolar materials and solvents have been successfully employed in the coating fluid.

Preliminary investigations indicate the described process to be suitable for applying rounding coats for sugar-coated tablets, film type coats to compressed tablets, and protective coats on small particles to prevent chemical decomposition. The process also appears to be useful for applying coating materials for the purposes of controlling drug release, improving flow properties of small particles, preparing tablet granulations, and many other coating operations in the pharmaceutical, food, animal feed, and other industries

Synthesis and Antifungal Studies on Sorbic Acid Derivatives*

By DALE H. CRONK†, LOUIS C. ZOPF, and JAMES W. JONES

Nine esters, one amide, and two amine salts of sorbic acid were prepared and tested against four pathogenic fungi by two methods. Of the twelve compounds, *o*-hydroxyphenyl sorbate, chlorothymol sorbate, isobutylamine sorbate, and morpholine sorbate exhibited the greatest activity.

Many compounds have been prepared and tested for antifungal activity. At the onset, the greatest interest was centered on compounds which would protect agricultural products against fungi which attacked the living plants or the crops derived therefrom to produce diseased plants or spoilage in the crop. In more recent years, attention has been given to compounds for the control of fungal-incited disorders in man and other animals. Among the compounds investigated are the fatty acids and their salts or organic derivatives. Rigler and Greathouse (1) studied the antifungal properties of fatty acids in connection with the resistance of plants to certain fungal diseases. They found that antifungal activity increased as the length of the carbon chain increased up to C_{11}, after which activity decreased. Geigy (2) reported that certain amides of α,β-unsaturated acids possess antifungal activity. Further studies on unsaturated acids led to the use of salts of undecylenic acid in the treatment of athletes foot and other dermatoses caused by fungi.

Sorbic acid, which is intermediate in the fatty acid series in respect to carbon chain length and is unsaturated at the α,β and γ,δ-positions, has been approved for the prevention of certain mold growths in foods, beverages, and cosmetic products. Since little work has been reported on the use of sorbic acid or its derivatives in the control of fungi which produce dermatoses or internal disorders in humans, the purpose of this investigation was to prepare certain derivatives and submit them to a preliminary screening against four strains of fungi which are known to be pathogenic. The fungi used were: (*a*) *Microsporum canis*, which is responsible for approximately 10 per cent of the cases of ringworm of the scalp (tinea capitis) among children in the United States. It also attacks glabrous skin. (*b*) *Microsporum gypseum*, which can produce tinea capitis and/or ringworm of the glabrous skin, but is not a common causative agent. (*c*) *Microsporum audouini*, the chief causative agent of tinea capitis among children. It also attacks glabrous skin. (*d*) *Trichophyton rubrum*, the most common cause of chronic dermatophytosis of the hands, feet, nails, and glabrous skin.

EXPERIMENTAL

Synthesis.—The esters and the anilide of sorbic acid[1] were prepared in the usual manner by the action of sorbyl chloride on certain phenolic compounds and aniline. The morpholine and isobutylamine salts were prepared by reacting sorbic acid with the amines. Table I shows the derivatives prepared along with their melting points, yields, and analytical data.

Antimycotic. *Method A.*—The sorbic acid derivatives were tested for antimycotic activity against *Microsporum canis*, *M. gypseum*, *M. audouini*, and *Trichophyton rubrum*. Spore suspensions for inoculation of the plates were prepared by removing pure colony growths from an agar slant and placing them in a 2-oz. wide-mouth bottle. Small pieces of glass rod and 20 ml. of water for injection were added and the bottle was shaken vigorously to disperse the spores. The agar plates were then inoculated with 0.5 ml. of this spore suspension.

The first series of plates were prepared by adding 20 ml. of the following medium, pH 6.2, to each plate: Glucose, 10.0 Gm.; Peptone, 2.0 Gm.; KH_2PO_4, 0.5 Gm.; $MgSO_4$, 0.5 Gm.; Agar, 15.0 Gm.; and Water, *q. s.* 100.0 ml. Two plates per organism per compound were used.

The test compounds, with the exception of morpholine sorbate, were dissolved in acetone in 5% concentrations. Water was used to dissolve morpholine sorbate since it was not soluble to the extent of 5% in acetone. Four drops of the solutions were placed on 12.7-mm. paper disks which were placed in the center of the inoculated plates. The zones of inhibition in mm. were measured after the plates had been incubated for fourteen days at room temperature. These data are recorded in Table II.

Another modification was that the seed cultures were grown in Petri dishes. When the colony had nearly covered the agar surface, 5-mm. squares were cut from an active growth area and transferred to the centers of freshly prepared agar plates. Four disks, impregnated with a compound as described above, were placed equidistant from each other at the periphery of the agar bed. The plates were then incubated at room temperature for fourteen days, after which the total colony growth was meas-

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[1] Supplied through the courtesy of Carbide and Carbon Chemicals Co., Division of Union Carbide Corporation.

TABLE I —ESTERS, ANILID, AND AMINE SALTS OF SORBIC ACID

No	Ester or Amine Component	Yield, %	M p, °C	C, Calcd	C, Found	H, Calcd	H, Found	N, Calcd	N, Found
1	*p*-Chlorophenol	53	64–66	64 86	64 97	4 95	5 21		
2	2,4-Dichlorophenol	65	73–75	56 25	56 09	3 90	4 07		
3	2,4,5-Trichlorophenol	16	100–102	49 65	50 10	3 10	3 28		
4	*p-tert* Butylphenol	36	56–58	78 68	78 49	8 19	7 93		
5	Methyl *p*-hydroxy benzoate	70	91–93	68 29	67 87	5 69	5 41		
6	Hexylresorcinol	5	95–97	75 00	75 86	8 33	8 62		
7	*o*-Hydroxyphenol	28	120–123	70 58	71 13	5 88	6 29		
8	Chlorothymol	34	Viscous liquid	69 06	67 58	6 83	6 94		
9	*o*-Chloro-*m*-cresol	65	49–51	61 10	66 11	5 50	5 56		
10	Aniline	49	150–153	77 00	77 65	6 95	6 76	7 48	7 19
11	N-Isobutylamine	79	[a]	64 86	64 84	10 27	10 06	7 56	7 33
12	Morpholine	96	131–136 with decompn	60 30	60 75	8 54	8 69	7 03	7.11

[a] No definite m p, gradually liquefied from 80–90°

TABLE II —ZONES OF INHIBITION IN MM, COMPOUNDS AT 5% W/V CONCENTRATION[a]

Compound No	Organism *M canis*	*M gypseum*	*M audouini*	*T rubrum*
1	19	21	19	17
2	21	18	27	19
3	19	16	30	23
4	17	14	16	19
5	0	0	0	0
6	0	17	20	19
7	21	24	26	31
8	50	40	83	54
9	20	18	23	24
10	0	20	16	15
11	18	14	29	29
12	23	0	20	35
Sorbic acid	33	33	41	37

[a] Average of four measurements from disk to edge of growth

ured in mm An average of four diameter measurements was recorded See Table III for these data

TABLE III —WIDTH OF COLONY GROWTH IN MM DIAMETER, COMPOUNDS AT 5% W/V CONCENTRATION

Compound No	Organism *M canis*	*M gypseum*	*M audouini*	*T rubrum*
1	38	59	48	44
2	66	64	56	54
3	57	65	[a]	55
4	60	56	52	48
5	55	54	[a]	59
6	65	59	53	42
7	60	64	[a]	48
8	36	51	21	37
9	61	54	48	50
10	62	65	57	65
11	59	53	48	40
12	59	54	46	47
Sorbic Acid	38	65	32	36

[a] Plate contaminated
For identification of compounds see Table I

Method B. This method consisted of streaking 0.5 ml. of spore suspension over the surface of a Sabouraud's agar plate. Solutions of the compounds were incorporated into hydrophilic ointment in 0.3 mole/Kg concentration based on the sorbate ion content of the molecule. The ointments were placed in collapsible tubes having an orifice about 2 mm in diameter Strips of the ointments, 5 cm long and 2 cm apart, were placed on the inoculated plates Zones of inhibition were determined after incubating the plates for nine days at room temperature. The zones were recorded in square mm They were determined by placing graph paper, divided into 6 3-mm squares, under each plate and counting the number of squares in the clear areas These results are shown in Table IV.

TABLE IV —ZONES OF INHIBITION IN NUMBER OF SQUARE MM OF AREA, COMPOUNDS TESTED AT 0 3 MOLE SORBATE/KG OF BASE

Compound No	Organism *M canis*	*M gypseum*	*M audouini*	*T rubrum*
1	238	647	834	540
2	66	476	687	437
3	0	0	0	250
4	0	304	302	449
5	0	0	0	222
6	132	159	488	357
7	331	953	1,322	913
8	1,255	1,322	1,759	1,170
9	699	1,026	1,326	993
10	238	476	461	647
11	1,255	1,945	1,957	1,719
12	1,032	1,771	1,957	1,532
Sorbic Acid	913	1,429	2,461	1,191

DISCUSSION

The results of method A as shown in Tables II and III indicate that only chlorothymol sorbate was more effective than sorbic acid against the four organisms used When method B was employed, the activity of the compounds was generally greater with chlorothymol sorbate, *p*-chloro-*m*-cresyl sorbate, isobutylamine sorbate, and morpholine sorbate exhibiting the best activity. The difference in the activities shown in methods A and B could be due to the fact that the ointments were prepared on the basis of moles of sorbate ion per 100 Gm of ointment rather than the w/v percentages, based on the entire compound used in preparing the solutions; hence, more drug was present to inhibit organism growth.

Also, the hydrophilic base may have aided in the dispersion of the drugs through the agar medium which is also aqueous. This factor might be especially true in the case of the water-soluble isobutylamine and morpholine sorbates.

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Binding of Drugs by Plastics I*

Interaction of Bacteriostatic Agents With Plastic Syringes

By E. MARCUS, H. K. KIM, and J. AUTIAN

A preliminary study was undertaken to ascertain if plastic syringes would bind certain bacteriostatic agents having acidic hydrogens. Three types of plastic syringes (nylon, polyethylene, and polystyrene) were included in the investigation. The results indicated that only the nylon syringe had any effect on the agents studied. Binding occurred within a relatively short contact period and was temperature dependent.

In the past several years, in this country and even more in Europe, more and more plastic appliances and devices have been introduced into medical and pharmaceutical practice. These plastic materials are replacing glass, rubber, and metals in such devices as tubings, containers, syringes, etc.

Unfortunately, the wide acceptance of plastics in other fields has created the illusion that plastics are inert and safe for any and all purposes. Some of this thinking has diffused into the medical and pharmaceutical field. There is at present, however, some concern by responsible representatives of the medical profession, pharmaceutical industry, plastic industry, and governmental agencies that certain plastic materials may be harmful, depending upon their use.

Part of the difficulty of recognizing that plastics may create some unfortunate problems with medicinal products is that many times information on a sample of plastic such as polyethylene, polystyrene, etc., will not always apply to another plastic with the same generic name. This may be understood if one considers that numerous formulations are available for the same generically named plastic. For example, one plastic manufacturer states that over two hundred formulations are used to produce polyvinyl chloride tubing.

Literature (1–5) contains much information concerning the acute and chronic toxicity of polymers and various additives upon animals. Less information (6) is available on chronic toxicity in humans. Unfortunately, only meager information is found on the effect of the plastic on a drug system or the effect of the drug system on the plastic.

One of the chief problems encountered with polyethylene is that it is pervious to gases and volatile substances (7). For example, volatile oils in certain pharmaceutical and cosmetic products have penetrated through the walls of the container, causing a change in aroma of the product (8). Permeation of air through the walls of the polyethylene containers has produced a change in color and taste of tetracycline suspension (8). Emulsion stability has been noted to decrease on storage in a plastic container as compared to storage in a glass container (9). Certain oils, such as mineral oil, myrrh, and Nigerian balsam deformed polyethylene bottles over a period of time (8). Sprays consisting of a combination of phenylephrine hydrochloride and thenylpyramine hydrochloride produced a brown precipitate when stored in polyethylene bottles. Clear, colorless polystyrene containers developed a cloudy appearance when exposed to certain fixed oils for several days (9).

Aqueous solutions of pentylenetetrazol have exerted a solvent action on several types of plastics (10). Various other parenteral products when exposed to polyvinyl chloride tubings were found to release acidic constituents to the solution (9). In certain instances, the plastic tubings caused a color change in the parenteral product. Leaching occurred with

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several types of plastic tubings, indicating that one or more of the additives in the tubing were released to the solution, depending upon the drug system exposed

In the evaluation of certain types of plastic disposable barrels (11), it was found that paraldehyde injection dissolved polystyrene barrels within five hours Dimercaprol injection produced an etching and clouding on the surface of polystyrene barrels but did not affect polyethylene or nylon barrels (11) A parenteral product containing diethyl carbonate as a solvent immediately dissolved a plastic hub of a disposable hypodermic needle (12)

The above review indicates that incompatibilities may occur which are easily discernible It is also obvious that when a visible physical change has occurred in a drug system or the plastic, the medical practitioner will certainly refrain from the use of that medication If the incompatibility is not so obvious, then the practitioner or nurse will not be alerted as to the consequence of the medication

Certainly, no one will question the importance of leaching of a substance from the plastic device into an injectable product, but of equal importance to the clinician is the realization that the drug product contains the full strength of the prescribed medication after contact with plastic devices such as tubings or syringes

From a review of the chemistry of a number of polymers utilized in plastics the authors speculated that certain polymers could conceivably bind a medicinal agent Furthermore, it was felt that those polymers containing charged negative centers, such as carbonyl groups, could react with proton donating agents forming an intermolecular complex In the past few years, a number of such intermolecular reactions have been reported for macromolecules and those chemical agents having acidic hydrogens (13–18)

In order to ascertain if certain types of plastics would appreciably absorb or bind certain medicinal agents, this preliminary study was initiated The experiment was designed to include three types of plastic barrels previously employed (11) in an incompatibility study The three plastic barrels were nylon, polyethylene, and polystyrene

EXPERIMENTAL

Apparatus and Reagents.—Beckman DU spectrophotometer, Beckman pH meter, model G, and constant temperature ovens Parahydroxybenzoic acid, Eastman Organic Chemicals, methylparaben U S P Heyden Chemical Corp , propylparaben U S P , Heyden Chemical Corp , sorbic acid Eastman Organic Chemicals phenol (C P) Allied Chemical & Dye Corp , and 4 chloro 3 methylphenol, Eastman Organic Chemicals

Plastic syringes (nylon, polyethylene, and polystyrene) supplied through Becton, Dickinson & Co , Rutherford, N J

2,6 Dibromoquinone chloroimide reagent solution 2,6 Dibromoquinone chloroimide, Eastman Organic Chemicals (80 mg of reagent dissolved in 25 ml of acetone free ethyl alcohol) *Buffer solution, pH 8 3* Boric acid, 12 369 Gm , potassium chloride, 14 911 Gm sodium hydroxide, 1 600 Gm , and distilled water to make, 1000 ml (When 5 ml of 1% sodium hydroxide is added to 10 ml buffer solution, a pH of 9 8 is obtained)

Methods of Analysis—Each bacteriostatic agent was recrystallized twice according to standard chemical procedures and the melting point determined to insure a relatively pure compound

Utilizing the procedure suggested by Singer and Stern (19), a colorimetric method of analysis was employed for the determination of phenol in aqueous solutions

Essentially, the procedure involves the production of a color by the addition of 2,6 dibromoquinone chloroimide reagent solution to phenol in a 1% sodium hydroxide solution The solution is then adjusted to a pH of 9 8 with boric acid sodium hydroxide buffer system After fifteen minutes the absorbance of the solution is measured at 600 mμ in a spectrophotometer In all instances, the solution to be assayed is adjusted to contain 8 μg or less of phenol

The other five agents were assayed by normal spectrophotometric methods of measuring the absorbance of the solutions in the ultraviolet region at the wavelength giving maximum absorption For each agent the maximum absorption and concentration are included in Table I Each of the agents conformed with the Beer Lambert law within the range of concentrations employed

TABLE I —WAVELENGTH OF MAXIMUM ABSORPTION AND CONCENTRATION USED IN CELL

Bacteriostatic Agent	Wave length mμ	Concn mg /100 ml
Parahydroxybenzoic acid	250	0 8
Methylparaben	256	0 8
Propylparaben	256	0 8
Sorbic acid	256	0 8
4 Chloro 3 methylphenol	280	4 0

Binding as a Function of Concentration and Temperature—Only the barrels of each type of syringe were employed in this study The metal canulas were removed from the syringes and the tips at the junction of the canula and the barrel were fused by the application of heat All of the barrels in the series were of 2 ml capacity

Four concentrations (except for phenol) for each agent were prepared using double distilled water as the solvent To dissolve the propylparaben and the sorbic acid, it was first necessary to dissolve these two agents in a small quantity of alcohol Each solution was then placed into each type of plastic barrel and stoppered with a suitable

plug. The plugs were composed of rubber stoppers encased in a strip of Parafilm[1] and tightly screwed into the open end of the barrel to minimize vaporization of the solution. It was found that even after forty-eight hours at 50°, the volatilization was not significant to markedly affect the assays.

The barrels were then stored at three different temperatures (5, 30, and 50° ± 1°), taking particular care not to have the solutions in contact with the stoppers. For each concentration, triplicate samples were employed as well as a control sample stored in a Pyrex test tube. After forty-eight hours of storage, the barrels were removed and the solutions from the triplicate samples pooled. These pooled samples were then assayed for their specific agents. Results were calculated as the average value for one barrel.

The results of this experiment indicated that only the nylon barrels bound any of the agents studied. For this reason, only the data of the nylon barrels were included in this paper. Tables II–VII include these results calculated as agent bound to the nylon. The data from the tables are plotted for each agent as bound agent *vs.* unbound agent and are shown in Figs. 1–6.

Binding as a Function of Time.—Another experiment was conducted to determine the extent of binding over a period of one week. In this experiment, solutions of the bacteriostatic agents were prepared at their usual pharmaceutical concentrations and placed into each type of barrel as described previously. Enough samples were prepared (in duplicate) to insure an adequate supply for the various determinations over the one week period. The results of this study are included in Table VIII and Fig. 7.

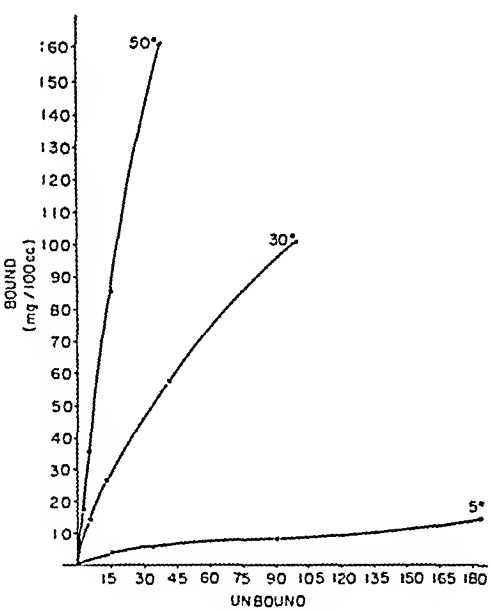

Fig. 1.—Binding of parahydroxybenzoic acid by nylon syringes.

[1] Trademarked product of Marathon Corp., Menasha, Wis.

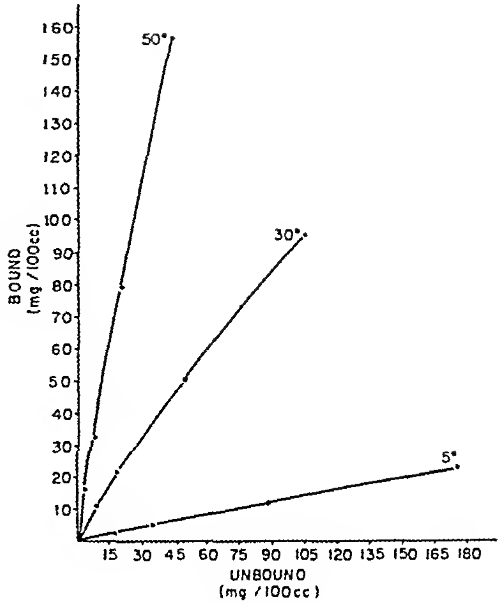

Fig. 2.—Binding of methylparaben by nylon syringes.

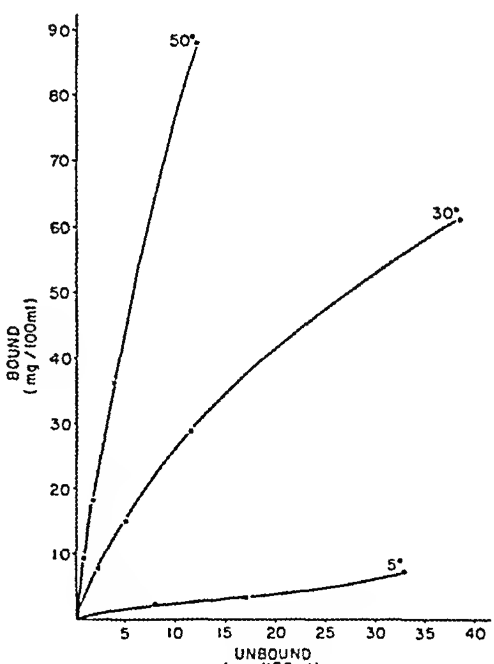

Fig. 3.—Binding of propylparaben by nylon syringes.

Each of the barrels above (after the one-week period) was dried with a clean, lint-free cloth and distilled water was placed into it. After forty-eight hours' storage at room temperature, the solutions were analyzed for the bacteriostatic agent originally present. In all instances, there was desorption occurring. No further study was continued on this desorption experiment.

TABLE II —BINDING OF PARAHYDROXYBENZOIC ACID BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C			30°C			50°C		
Concn %	Bound mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound, %
0 20	15 1	7 6	0 20	100 7	50 4	0 20	161 5	80 8
0 10	8 6	8 6	0 10	57 2	57 2	0 10	84 9	84 9
0 04	6 9	17 3	0 04	26 7	66 8	0 04	34 7	86 8
0 02	4 2	21 0	0 02	14 2	71 0	0 02	17 4	87 0

TABLE III —BINDING OF METHYLPARABEN BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C			30°C			50°C		
Concn , %	Bound, mg /100 ml	Bound %	Concn , %	Bound, mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound %
0 20	23 3	11 7	0 20	95 4	47 7	0 20	156 2	78 1
0 10	12 5	12 5	0 10	50 0	50 0	0 10	79 2	79 2
0 04	5 0	12 5	0 04	21 5	53 8	0 04	32 3	80 8
0 02	2 3	11 5	0 02	11 0	55 0	0 02	16 1	80 5

TABLE IV —BINDING OF PROPYLPARABEN BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C			30°C			50°C		
Concn %	Bound mg /100 ml	Bound, %	Concn %	Bound, mg /100 ml	Bound, %	Concn , %	Bound mg /100 ml	Bound %
0 10			0 10	61 3	61 3	0 10	88 0	88 0
0 04	7 2	18 0	0 04	28 7	71 8	0 04	36 2	90 5
0 02	3 2	16 0	0 02	14 9	74 5	0 02	18 3	91 5
0 01	2 1	21 0	0 01	7 7	77 0	0 01	9 2	92 0

TABLE V —BINDING OF SORBIC ACID BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C			30°C			50°C		
Concn , %	Bound mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound, %
0 20	9 8	4 9	0 20	53 8	26 9	0 20	113 8	56 9
0 10	7 8	7 8	0 10	33 4	33 4	0 10	65 5	65 5
0 04	2 8	7 0	0 04	15 7	39 3	0 04	26 5	66 3
0 02	1 6	8 0	0 02	8 1	40 5	0 02	13 6	68 0

TABLE VI —BINDING OF PHENOL BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C			30°C			50°C		
Concn , %	Bound, mg /100 ml	Bound, %	Concn , %	Bound mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound, %
5 00	1750 0	35 0	5 00	3000 0	60 0	5 00	3500 0	70 0
2 00	100 0	5 0	2 00	900 0	45 0	2 00	1300 0	65 0
1 25	62 5	5 0	1 25	565 0	45 2	1 25	812 5	65 0
1 00	50 0	5 0	1 00	450 0	45 0	1 00	650 0	65 0
0 50	25 0	5 0	0 50	225 0	45 0	0 50	350 0	70 0

TABLE VII —BINDING OF 4-CHLORO-3-METHYLPHENOL BY NYLON BARRELS AS A FUNCTION OF CONCENTRATION AND TEMPERATURE

5°C			30°C			50°C		
Concn , %	Bound mg /100 ml	Bound, %	Concn , %	Bound mg /100 ml	Bound, %	Concn , %	Bound, mg /100 ml	Bound, %
0 40	240 0	60 0	0 40	346 0	86 5	0 40	379 2	94 8
0 20	65 0	32 5	0 20	170 0	85 0	0 20	187 6	93 8
0 10	25 0	25 0	0 10	85 0	85 0	0 10	93 8	93 8
0 05	11 3	22 6	0 05	42 5	85 0	0 05	46 4	92 8

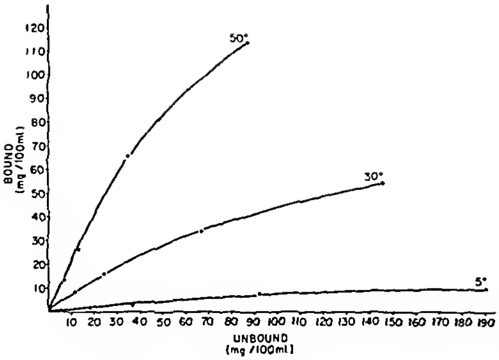

Fig. 4.—Binding of sorbic acid by nylon syringes

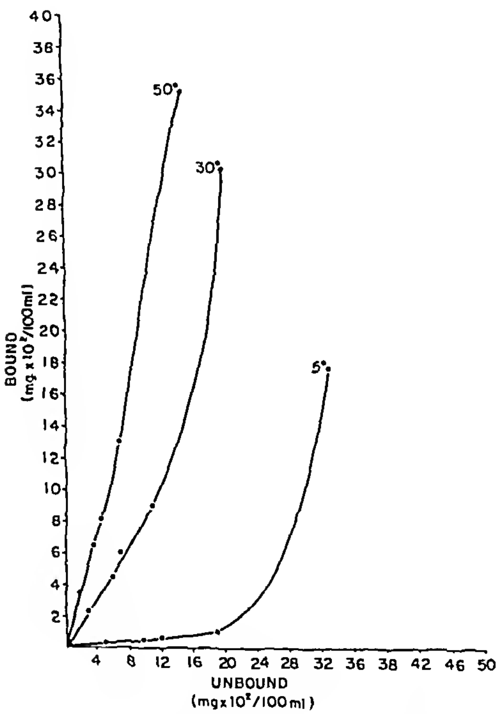

Fig. 5.—Binding of phenol by nylon syringes.

Table VIII.—Per Cent of Agent Bound by the Nylon Barrels at 30°

Drug	Initial	5 hr	24 hr	48 hr.	1 wk
Parahydroxybenzoic acid (0.2%)	0	14.4	31.6	50.4	78.7
Methylparaben (0.2%)	0	15.3	34.1	47.7	75.5
Propylparaben (0.1%)	0	22.2	44.4	61.3	85.1
Sorbic acid (0.2%)	0	7.1	13.7	26.9	47.0
Phenol (2.0%)	0	16.9	30.7	47.4	60.5
4-Chloro-3-methylphenol (0.2%)	0	8.4	52.6	59.6	85.5

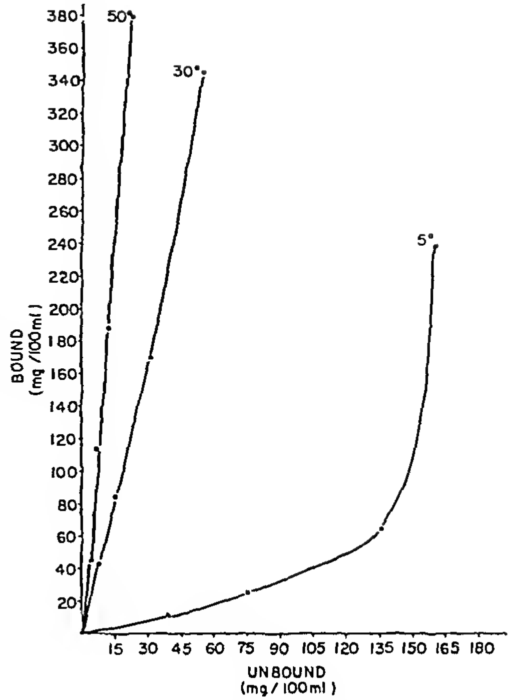

Fig. 6.—Binding of 4-chloro-3-methylphenol by nylon syringes.

RESULTS AND DISCUSSION

Analysis and Equilibrium State.—The analysis of the pooled solutions from three barrels was used in the experiment because it was found that there could be variations in barrel action from one syringe to another. In most instances the difference was small, but occasionally there was a large discrepancy in analytical results from one nylon barrel to another nylon barrel, even though both were distributed by the same source. Also, the pooled samples could be handled more efficiently than the individual samples from one barrel.

Initially, it was felt that forty-eight hours of contact would be sufficient time for equilibrium to be reached, but this did not prove to be the case. Preliminary experimentation on this problem at 30° indicated that a state of equilibrium would not be reached even after several weeks of contact

Binding as Function of Concentration and Temperature.—In this study the exact mechanism of interaction was not determined, however, it was speculated that the carbonyl groups of the polyamides acted as negative charged centers attracting the proton donating groups of the phenols, the main forces of bonding being through hydrogen bonds Patel and Kostenbauder (17) have noted this type of interaction when nylon dialysis bags were used in a binding study of certain phenolic preservatives. Pakshver and Mankash (18) postulated that phenols will bind to nylon by a dipole-dipole interaction.

The plots in Figs. 1–6 had a relatively linear relationship at the lower concentration levels As the concentration levels of the agents (except phenol and 4-chloro-3-methylphenol) were increased the

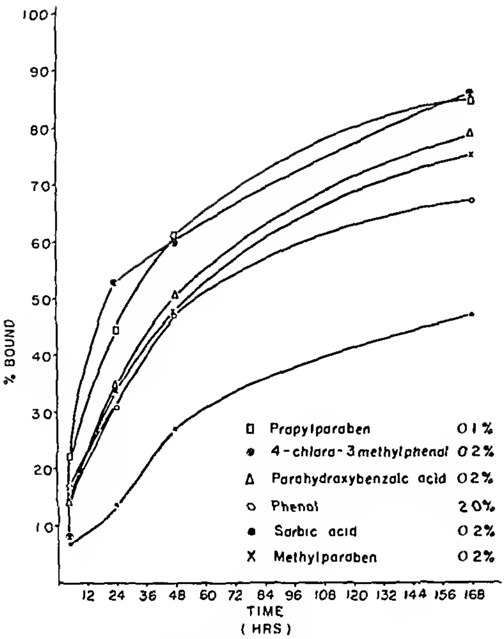

Fig 7—Binding of drugs by nylon syringes as a function of time

slopes of the lines decreased. For a first approximation these curves resemble a Langmuir adsorption isotherm In dilute solutions, the molecules of the drug are not hindered in approaching the binding sites on the surface and within the nylon, but as the concentration is increased, there is hindrance to the approach of the molecules for the remaining sites.

The above relationship is not seen in Fig. 6 or 7 for the two phenols. In this case, especially observed at 5°, there is an abrupt inflection and increase in the slope (increase in binding) of the line. One explanation for the anomalous results observed for the two phenols is based upon the fact that as the phenolic solution diffuses within the nylon, there is an alteration in the nylon structure (reduction of tensile strength (20)). However, there appears to be a critical concentration level where the solution can literally "break-through" the polymer matrix Once this has occurred, a great many more sites are available.

It is also obvious from all the figures that as the temperature is increased, the binding phenomenon increases. This probably is explained on the basis that the rate of diffusion is increased and, consequently, more sites in the polymer become available for the drugs to be bound.

Binding as Function of Time.—Table VIII and Fig. 7 show the relationship between time of contact and degree of binding. Even after only five hours of contact at 30°, significant amounts of each bacteriostatic agent were bound. After one week, in nearly all instances, over 60% of the agents were bound.

Significance of Study. Perhaps the real importance of this preliminary study is the realization that plastic materials may not be inert to drug systems Even though only six bacteriostatic agents were employed in this study, it seems likely that other substances may behave in the same way with nylon or other plastic materials having polar groups.

SUMMARY

1. A study was conducted to determine if certain types of plastic syringes would bind six bacteriostatic agents.

2. The nylon barrel bound to various degrees parahydroxybenzoic acid, methylparaben, propylparaben, sorbic acid, phenol, and 4 chloro-3-methylphenol.

3. Polyethylene and polystyrene barrels did not indicate any tendency to bind the six bacteriostatic agents reported in this study.

4. The degree of binding for each agent was influenced by concentration, temperature, and diffusion.

5. Further research is being conducted on this type of problem.

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Influence of Glutathione on the Inhibition of *Escherichia coli* by Kanamycin*

By ROBERTSON PRATT and YOKO YUZURIHA

The tolerance of *Escherichia coli* ATCC 6522 for kanamycin sulfate was increased from 5 μg./ml. to 15,000 μg./ml. in 63 serial passages through a chemically-defined medium containing increasing concentrations of the antibiotic. Glutathione was shown to interfere with the growth-inhibiting effects of kanamycin on *Escherichia coli* when the kanamycin concentration was 5 μg. or less per ml. and the molecular ratio of kanamycin to GSH was approximately 16 or less.

The ability of *Escherichia coli* to survive and multiply in the presence of potassium benzyl penicillin may be increased or decreased by the addition of glutathione (GSH). The direction and the magnitude of the change depend on the absolute concentration of penicillin and on the relative concentrations of the antibiotic and the GSH with respect to each other (1). Apparently the complete GSH complement is required to produce the phenomenon, since the constituent moieties of GSH, whether supplied separately or together in the same ratio as in the complete molecule, do not reproduce the effect (2).

We have become interested in extending these observations to other antibiotics. The present report is concerned with the interaction of components in the *E. coli*-GSH-kanamycin system, as expressed through ability of the organism to multiply in a chemically defined nutrient solution containing either or both of the compounds.[1]

Kanamycin was isolated in Japan in 1957 from culture filtrates of *Streptomyces kanamyceticus* (3) and was developed industrially in the United States by the Bristol Laboratories who began to market the sulfate, under the trade name Kantrex, in 1958. Some of the basic and clinical research that preceded marketing of the drug has been collected in a single publication (4).

EXPERIMENTAL

Methods.—*Escherichia coli* ATCC 6522 was maintained as in the work reported previously (1, 2) by daily transfers in Anderson's chemically defined medium as modified by Cowie, *et al.* (5). Inocula for experiments were taken from cultures incubated at 37° for eighteen hours. Organisms were centrifuged and washed three times in sterile distilled water and finally were resuspended to give a transmission of from 46 to 48%, as determined in Pyrex tubes (18 mm., i. d.) in a Lumetron, Model 402 EF, equipped with a neutral filter. One milliliter of this suspension inoculated into 14 ml. of medium provided a suspension with an initial optical density of 0.02 to 0.025 and an initial viable count of approximately 200×10^6 organisms per ml. Phosphate buffer was used to adjust culture solutions to pH 6.8. Cultures were incubated in inclined roller tubes rotating at a rate of 5 r. p. m.

Solutions of glutathione and of kanamycin were sterilized by filtration through sintered glass. All other components of the medium were sterilized in an autoclave.

Results.—*Minimum Inhibitory Concentration.*—The MIC for the parent organism was between 5 and 10 μg. of kanamycin per ml. in the medium that was employed. At a concentration of 5 μg./ml., there was a small initial burst of growth during the first five hours, but two hours later the cultures were visually clear and remained so for twenty-four hours (Fig. 1). However, growth equal to that of control cultures occurred in forty-eight hours. No growth occurred, even after prolonged incubation, when the organisms were inoculated into media containing 10 μg. of kanamycin per ml.

Resistance.—Upon transfer of organisms that had grown out in forty-eight hours in culture medium containing 5 μg./ml. to fresh solutions containing the same concentration of drug, full growth occurred in twenty-four hours or less. Subculturing these organisms in increasing concentrations of antibiotic yielded, in 63 transfers, a strain that produced full growth in twenty-four hours in medium containing 15,000 μg. kanamycin per ml. Full growth we define as growth equivalent to that of the parent strain (never exposed to the antibiotic) in twenty-four hours in the standard antibiotic-free medium.

Effect of Glutathione.—All experiments with GSH were conducted with the parent strain of *E. coli* which had not been exposed previously to kanamycin. Maximum growth of the organism was not affected by addition of from 0.02 to 200 μg. of GSH/ml. to the antibiotic-free culture medium. The rate of growth during four to five hours was slightly accelerated when the medium contained 20 μg. or 200 μg. of GSH/ml. but not when it contained lesser concentrations.

In culture solutions containing 1 μg. of kanamycin per ml. there was some reduction of growth during the first few hours after inoculation but no significant effect after twenty-four hours (Fig. 1). Supplied at an initial concentration of 2 μg./ml., kanamycin caused substantial retardation of growth for at least seven hours and an appreciable reduc-

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[1] The kanamycin used in this work was kindly provided by the Bristol Laboratories.

tion even at the end of twenty-four hours However, these effects were eliminated when as little GSH as 0.02 μg./ml. ($6.5 \times 10^{-8}M$) was added to the medium, and growth curves for these cultures and those containing larger amounts of GSH were the same as for antibiotic-free control cultures provided with the corresponding concentrations of additive

Addition of GSH at a level of 0.2 μg./ml. ($6.5 \times 10^{-7}M$) partially reversed the inhibitory action of kanamycin supplied at a concentration of 3 μg./ml. Lower concentrations of GSH had no effect As the concentration of GSH was increased to 2.0 and then to 20 μg./ml., there was progressive diminution of the inhibitory action of the antibiotic during the first seven hours of growth, and after twenty-four hours the optical density of cultures initially containing 3 μg. of kanamycin and from 0.2 to 20 μg. of GSH per ml. was equal to that of the control culture (Fig 2)

At kanamycin levels of 4 μg. and 5 μg./ml., addition of GSH also partially reversed the action of the antibiotic (Fig 2). No growth occurred, even upon prolonged incubation, in media containing 10 μg. of kanamycin per ml., irrespective of the concentration of GSH.

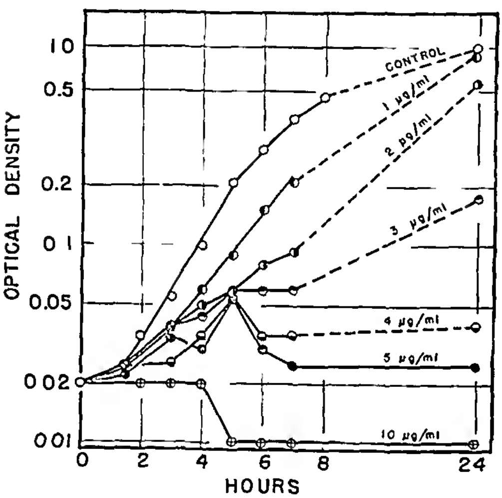

Fig. 1.—Growth of *Escherichia coli* ATCC 6522 in a chemically-defined medium containing the indicated concentrations of kanamycin

The approximate parallelism of the seven-hour curves for 3, 4, and 5 μg. of kanamycin per ml. (Fig 2) indicates that a given increase in GSH concentration was accompanied by an approximately equal (percentagewise) reduction in the inhibitory effectiveness of the antibiotic, irrespective of the concentration of the latter At GSH concentrations above 20 μg./ml., the curves flattened off The optical density at seven hours for cultures containing 200 μg. of GSH per ml. was not significantly different from that of cultures containing only 20 μg. of additive per ml.

A different situation obtained at twenty-four hours In cultures containing, respectively, 3, 4, and 5 μg. of kanamycin per ml., a given increase in GSH concentration was accompanied by progressively greater reduction in inhibitory effectiveness of the antibiotic as the concentration of the latter increased. This is indicated by the progressively increasing slopes of the curves for the increasing concentrations of antibiotic (Fig 2) As was found for the growth at seven hours, a GSH concentration of 0.02 μg./ml. had no effect on the total twenty-four-hour growth of organisms exposed to 3, 4, or 5 μg. of kanamycin per ml., and raising the GSH concentration to 200 μg./ml. had no significant effect beyond that associated with the 20 μg./ml. level

Calculations of molecular concentrations showed that, as a generalization, when the absolute concentration of kanamycin was in the range 3 to 5 μg./ml. (6.2×10^{-6} to 10.3×10^{-6} M) and the kanamycin GSH index (molecular concentration of kanamycin divided by molecular concentration of GSH) was of the order of approximately 16 or less, the effectiveness of the antibiotic against *E. coli* was diminished This was a purely empirical observation, and we do not mean at this time to suggest that biochemical implications with respect to mechanisms of action of kanamycin can be drawn from it

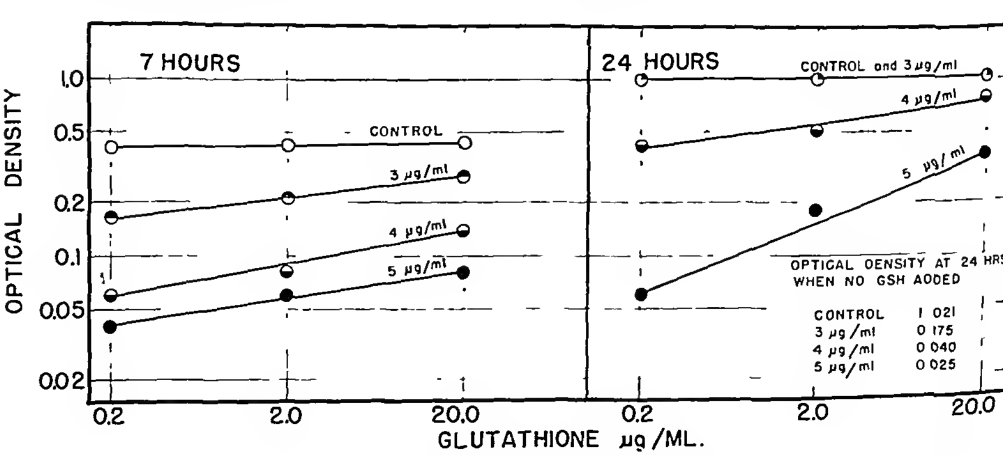

Fig 2.—The effect of increasing concentrations of glutathione on the inhibitory action of the indicated levels of kanamycin against *Escherichia coli* ATCC 6522

DISCUSSION

It remains to be determined whether inhibitory effects of GSH on the antibacterial action of kanamycin, similar to those described above, can be demonstrated with other organisms and in other media. Also remaining to be determined is the significance, if any, of the increasing inhibitory effect at twenty-four hours of a given increment of GSH concentration as the concentration of kanamycin was increased from 3 to 5 μg./ml. when no such effect could be observed at seven hours.

However, the observed data suggest interesting speculation concerning their possible clinical significance. Currently available data indicate that in adults a daily dosage regimen of 0.25 Gm. per eight hours can be expected to produce serum concentration of kanamycin ranging at different times from about 2.0 to 12.0 μg./ml. or that on a 0.5 Gm. per twelve-hour schedule a range of about 2.0 to 28.0 μg./ml of serum can be anticipated, depending on the time elapsed since the last previous injection and factors of patient variability (6). The figures are roughly compatible with data showing a range from 1.3 to 14 μg./ml. of serum in infants and children receiving a single intramuscular dose of 5 mg./Kg. (7). Assuming that serum accounts for approximately 60% of the total volume of whole blood (8) and that most, if not all, of the blood-kanamycin is in the serum, the above figures are comparable to whole blood levels ranging from less than 1.0 to about 17 μg./ml.

Blood or serum levels of antibiotics are not important in themselves, in the absence of bacteremia. Since the primary action of most antibiotics is exerted directly on the invading organisms and not indirectly, as for example, through effects on host defense mechanisms, it is the actual concentration of the drug at the sites where the pathogens are that is important in determining clinical effectiveness. But since it is not always possible or, if possible, convenient to determine concentrations at the specific sites of infection, it has become conventional to rely on the blood level as an index of therapeutic concentration. Justifiable as this practice is empirically, it is important to bear in mind that the blood level is simply a symbol and, even though it may bear a definite relationship to the antibiotic concentrations in various other body tissues and fluids, not to confuse it, in absolute terms, with the value for which it is merely an index. The figures cited in the preceding paragraph indicate that at certain periods during treatment with kanamycin the concentration of the antibiotic, as judged by the response of *E. coli*, falls within the range in which action of the drug is subject to interference by GSH. It seems entirely possible that the periods during which such concentrations prevail in other tissues and fluids may be reached sooner and last longer.

Reliable data are available on the GSH content of blood and of a few specific organs, e. g., the eye and the liver, but there seems to be a scarcity of data relating to body tissues or organs in general. However, tissue levels of GSH probably are usually at least as high as the *minimum* concentration (0.02 to 0.2 μg./ml.) shown above to interfere with the antibacterial action of low concentrations of kanamycin against *E. coli* in a synthetic medium.

The normal GSH content of human blood has been reported to be about 350 μg./ml. (9). In severe mental disease (schizophrenia, manic-depressive psychosis, and involuntional melancholia), the GSH index may be reduced as much as 40% (10). Likewise, "marked reduction in blood GSH" has been observed in ketonemia accompanying diabetes and in diffuse and severe liver disease. Even so, the GSH content of the blood is not likely to fall below 175 to 200 μg./ml. Presumably most of the GSH is in the formed structures (mostly erythrocytes) of the blood (9), but some may also be present in serum.

The inhibitory effect of GSH on the antibacterial activity of low concentrations of kanamycin may merit consideration in planning injection schedules aimed at achieving maximum clinical effectiveness of the drug.

SUMMARY

The effect of kanamycin on the growth of *Escherichia coli* ATCC 6522 in a chemically defined medium, with and without addition of glutathione (GSH), has been studied.

1. The minimum inhibiting concentration of kanamycin in the absence of GSH was between 5 and 10 μg./ml.

2. A 3,000-fold increase in tolerance was developed in 63 serial passages of the organism through increasing concentrations of the drug.

3. GSH in a concentration of 0.02 μg./ml. completely nullified the inhibitory effect of kanamycin supplied in concentrations of 1 or 2 μg./ml.

4. In a concentration of 0.2 μg. or more per ml., GSH partially or completely reversed the antibacterial action of kanamycin provided at 3, 4, or 5 μg./ml. The degree of reversal was a logarithmic function of GSH concentration between 0.2 and 20 μg./ml. and depended on the time at which observations were made.

5. Speculation on a theoretically possible clinical implication of the data is suggested.

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Saponins and Sapogenins IV*

Isolation of Oleanolic Acid from *Sesbania Aegyptica* Pers.

By M. O. FAROOQ, I. P. VARSHNEY, and MOHD. S. Y. KHAN

The seeds of *Sesbania Aegyptica* Pers have been found to contain two triterpenic sapogenins, one an acid sapogenin and the other a neutral one The acid genin has been identified as oleanolic acid by a direct comparison of the melting point and the infrared spectra of the methyl ester of the acetate with an authentic sample

Sesbania Aegyptica Pers, locally known as 'Ravasin, is a member of the family *Leguminosae*, sub family *Papilionatae*, and grows wild throughout the plains of India Owing to its rapid growth it is being increasingly used as a hedge plant and it is a good cattle feed The seeds are used in cases of the enlargement of the spleen (1) A number of the plants of the family *Leguminosae* have been found to contain saponins and sapogenins (2–5), but the only record about this plant is that it contains no saponin (6) During our work on the fat content (7) of the seeds of the plant, the presence of a saponin was indicated and therefore its systematic examination for the saponin content was taken up

The seeds of *Sesbania Aegyptica* Pers were procured from M/S N Cooper & Co, Poona as well as obtained from the plants grown on the University campus A quantity of the finely powdered defatted seeds were extracted with ethanol The recovery of the solvent left an oily residue which was successively treated with ether, petroleum ether, carbon tetrachloride, and acetone The residue thereafter was taken up in alcohol and precipitated by addition to a large amount of ether This precipitation was repeated a number of times This gave colorless saponin which became a brown syrup on exposure to atmosphere and satisfied all the tests for saponin The saponin was dissolved in a large amount of water and hydrolyzed with sulfuric acid The genin obtained was filtered and washed free of the acid The failure of a number of attempts at crystallization from different solvents suggested the genin to be a mixture and, therefore, it was refluxed with a solution of alcoholic potassium hydroxide and extracted with ether Evaporation of the ethereal layer gave a neutral genin while the alkaline solution, on treatment with an excess of hydrochloric acid gave a precipitate of an acid genin This acid genin gave an acetate which, on crystallization from methanol, had m p 261–263°, $[\alpha]_D = +72\ 7$ The acetate gave positive response to all the usual tests for a triterpene

The resemblance of the infrared spectrum of the acetate with the spectra of the acetates of the triterpenic acids of β amyrin group (methylene bending absorption) was very close and did not resemble at all the infrared spectra of the acetate of the steroidal sapogenins (finger print region) (2, 4)

The deacetylation of the acetate gave an acid genin, m p 285–288°, $[\alpha]_D = +80$ The analysis of the acetate indicated the presence of one hydroxyl and one carboxyl group in a pentacyclic triterpenic compound

The carboxyl function which was readily detectable in the infrared spectra was fixed by the formation of a methyl ester, m p 195–198° $[\alpha]_D = +76$ The fact that it was obtained with diazomethane and not with methanolic hydrochloric acid, coupled with the difficulty of its hydrolysis, suggested the attachment of the carboxyl group to a tertiary carbon atom (i e, to C 17) The methylation of the acetate with diazomethane gave an acetyl methyl ester, m p 217–219°, $[\alpha]_D = +72\ 7$ The genin and all its derivatives showed unsaturation with tetranitromethane

The genin on oxidation with chromic acid gave a ketone which gave a positive Zimmermann test (8) showing the position of the carbonyl group at C 3 The ketone gave a 2 4 dinitrophenylhydrazone without difficulty

The relation with the β amyrin group was further established by the examination of the U V spectrum of the product of oxidation of the acetate with selenium dioxide The product obtained on oxidation could not be crystallized and showed the characteristic triple ultraviolet absorption maxima (λ_{max}^{EtOH} 241, 249, 255 mμ) of a diene

By analogy with all the acids of the β amyrin group the position of one of the hydroxyl groups has been assumed to be at C 3 in ring A, and that of the carboxyl group at C 28 attached to C 17 The comparison of the physical constants of the

* Received April 5 1959 from the Department of Chemistry Muslim University Aligarh India
For earlier three parts see (5)
The authors are grateful to Prof T R Govindachari of the Presidency College Madras for the infrared spectra

genin and its derivatives with all the known acids of the β-amyrin group carrying one hydroxyl group indicates the present genin to be identical with oleanolic acid (Table I).

The comparison of the infrared spectra (Fig. 1) and mixed melting point of the acetyl methyl ester of the genin with authentic samples of acetyl methyl oleanolate obtained from *Albizzia lebbek* (2) and *Randia dumetorum* (9) confirmed the identity of the genin as oleanolic acid.

TABLE I.

	Oleanolic Acid m. p.	$[\alpha]_D$	Present Genin m. p.	$[\alpha]_D$
Genin	310	+80	285–288	+80
Acetyl oleanolic acid	268	+74.5	261–263	+72.7
Methyl oleanolate	198–200	+75	195–198	+76
Acetyl methyl oleanolate	219–220	+70	217–219	+72.7

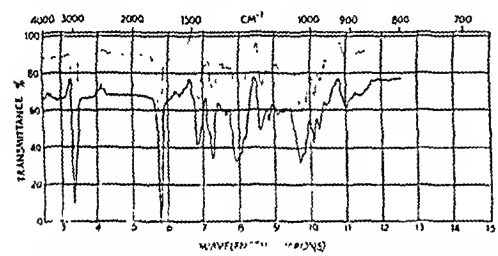

Fig. 1.—Comparison of infrared spectra. Solid line shows present genin acetyl methyl ester. Broken line shows acetyl methyl oleanolate.

EXPERIMENTAL

All the melting points recorded in this paper have been taken on a Kofler hot microscopical stage and are corrected. The infrared spectra have been taken in chloroform solution at the Presidency College, Madras, using a double beam Perkin-Elmer spectrometer Model 137 (Infracord) and interpreted by one of the authors (I. P. V.). The microanalyses recorded here have been done at the Department of Chemical Technology, University of Bombay. The ultraviolet spectra have been measured using a Beckman spectrophotometer Model DU.

Defatting.—Well-powdered seeds (1 Kg.) were exhausted in a Soxhlet extractor with light petroleum ether (40–60°). The recovery of the solvent left a greenish oil (55 Gm.). The exhausted seeds were dried before further operation.

Extraction.—The defatted seed powder was exhausted with 95% alcohol in a Soxhlet extractor and the solvent recovered leaving a brown syrupy liquid. The residual syrup was dissolved in ethanol and filtered. The alcohol was recovered and the solid residue extracted with ether, petroleum ether, carbon tetrachloride, and acetone, successively. This gave a brown syrupy mass, which was dissolved in a little alcohol and added to a large amount of ether, which precipitated the saponin. This process of dissolution in alcohol and precipitation by ether was repeated three times. It gave colorless saponin which turned to a syrup on exposure to air, and gave all the tests for saponin.

Isolation of Sapogenin.—The brown syrupy mass (25 Gm.) was dissolved in water (6 L.) and hydrolyzed with sulfuric acid (300 Gm.) by heating the solution first on a boiling water bath for an hour and thereafter completing the hydrolysis by boiling the solution for another hour. After about twenty to thirty minutes a precipitate began to appear which went on increasing until the hydrolysis was completed. It was then filtered and washed with water until the filtrate was neutral. It was dried in an air oven at 80°. The dried genin was dissolved in alcohol and decolorized with charcoal. All attempts at crystallization from various solvents proved fruitless.

Separation of Acid and Neutral Genin.—The crude genin (1 Gm.) was heated with alcoholic caustic potash (20 Gm. KOH in 300 cc. of alcohol) for a half-hour and then half the solvent was distilled off. The solution was then diluted with water (2 L.) and extracted three times with ether. The ethereal extracts were combined and washed free of the alkali. It was dried over sodium sulfate (anhydrous) and removal of the ether left a neutral substance in the flask.

The alkaline solution was acidified with hydrochloric acid when it gave a precipitate. This was filtered, washed free of the acid, and dried.

Acetylation.—The acid genin obtained as above was acetylated by treatment with pyridine and acetic anhydride in the cold for eighteen hours. It was poured into ice water and filtered. The precipitate was washed free of the acid and pyridine and crystallized twice from methanol to obtain the product in fine, colorless needles, m. p. 261–263°, $[\alpha]_D^{25} = +72.7$ (C = 0.165 $CHCl_3$), yield = 500 mg. It gave a positive test with tetranitromethane, $\lambda_{max.}^{CHCl_3}$ 5.8, 5.9, and 8.0μ.

Anal.—Calcd. for $C_{32}H_{50}O_4$: C, 77.11; H, 10.40. Found: C, 77.0; H, 9.8.

Deacetylation.—The acetate (250 mg.) was refluxed for two hours with 15 cc. of 5% methanolic potassium hydroxide. The solution was diluted with 200 cc. water and left overnight at room temperature. It did not yield any crystalline potassium salt. The solution was acidified with hydrochloric acid and the precipitate formed was washed free of the acid and crystallized from methanol, m. p. 285–288°, $[\alpha]_D^{26} = +80$ (C = 0.100 $CHCl_3$). It gave a positive test with tetranitromethane.

Selenium Dioxide Oxidation.—Acetate (100 mg.) in 15 cc. acetic acid was heated under reflux with 100 mg. freshly sublimed selenium dioxide for two hours. It was poured into water and extracted with ether. It could not be crystallized. $\lambda_{max.}^{EtOH}$ = 241, 249, 255 mμ.

Methylation.—The acid genin (200 mg.) was dissolved in 200 cc. ether and an excess of ethereal solution of diazomethane added. It was left overnight, the excess of diazomethane was removed on a water bath, and the product was crystallized from methanol, m. p. 195–198°, $[\alpha]_D^{26} = +76$ (C = 0.098 $CHCl_3$) The substance gave a positive test with tetranitromethane.

Anal.—Calcd. for $C_{31}H_{50}O_3$: C, 79.1; H, 10.6. Found: C, 78.2; H, 10.8.

Demethylation.—The methyl ester (100 mg.) was refluxed with 100 cc of 5% methanolic caustic potash for half an hour The starting material (80%) was recovered unchanged and only 20% of the ester was demethylated The genin obtained was confirmed by melting and mixed melting points

Acetyl Methyl Ester.—The acetate (100 mg) was dissolved in ether and an excess of a solution of a diazomethane added to it After a contact of twenty hours ether was removed and the residue crystallized from methanol, m p 217–219°, $[\alpha]^{26}_{D} =$ +72 7 (C = 0 165 $CHCl_3$) It gave a yellow color with tetranitromethane $\lambda^{CHCl_3}_{max} = 5\,8, 8\,0\ \mu$

Anal—Calcd for $C_{33}H_{52}O_4$ C, 77 29, H, 10 22 Found C, 77 3, H, 9 7

Oxidation of the Genin.—A solution of chromic acid (100 mg) in 15 cc of 80% acetic acid was added to 100 mg of the genin dissolved in 20 cc acetic acid After leaving it for half an hour at room temperature, 100 cc alcohol was added to destroy the excess chromic acid The solution was left for fifteen minutes and then the alcohol was distilled off under reduced pressure The residue was dissolved in aqueous alcohol and extracted with ether The ethereal solution was washed well with water and evaporated to dryness The product crystallized from methanol as fine needles, m p 156–160° It gave a positive Zimmermann test

2,4-Dinitrophenylhydrazone.—The ketone (50 mg) was dissolved in 10 cc alcohol and heated on a water bath It was then mixed with a solution of 50 mg 2,4-dinitrophenylhydrazine in 10 cc alcohol and 0 4 cc hydrochloric acid It was left at room temperature for five minutes when a precipitate was formed It was kept overnight, filtered, washed, and recrystallized from ethanol, m p 251–255°

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Studies on Cell Growth and Cell Division I*

A Temperature-Controlled System for Inducing Synchronous Cell Division

By KWAN-HUA LEE

A temperature-controlled system for inducing cell growth and synchronous cell division in *Tetrahymena pyriformis* is described. By using this device, it is possible to dissociate cell growth and cell division into two distinct processes.

RECENTLY, Scherbaum and Zeuthen (1) have demonstrated that when proteose-peptone cultures of the ciliate protozoon *Tetrahymena pyriformis* GL were incubated at alternate half-hour periods at 28° (optimum for growth) and 34° (sublethal) for six or seven cycles, the size of the ciliates was increased to about three times larger than the normal cells without any cell division taking place. When these heat-treated cells were brought back to 28°, either in a nutrient medium or in a nutrient-free buffer solution (2), they went into cell division synchrony for three times before normal random cell division was resumed In the nutrient medium, both cell division and cell growth took place while in the nutrient-free medium only cell division was possible. This technique dissociates cell growth and cell division into two distinct processes and thus provides biological material for the studies on selective activity of drugs on either process without any interference of the other process

The technique of inducing synchronous cell division has since been applied to other strains of *Tetrahymena pyriformis* (3) and many other forms of unicellular organisms (4). In this paper, a practical temperature-controlled system for inducing cell growth and cell division is described The principle applied in the design has many obvious advantages and it can also be adjusted to cycle at any other temperature levels at specified rate and intervals

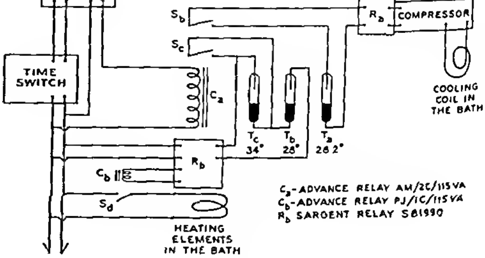

Fig 1 —Wiring diagram of the water bath

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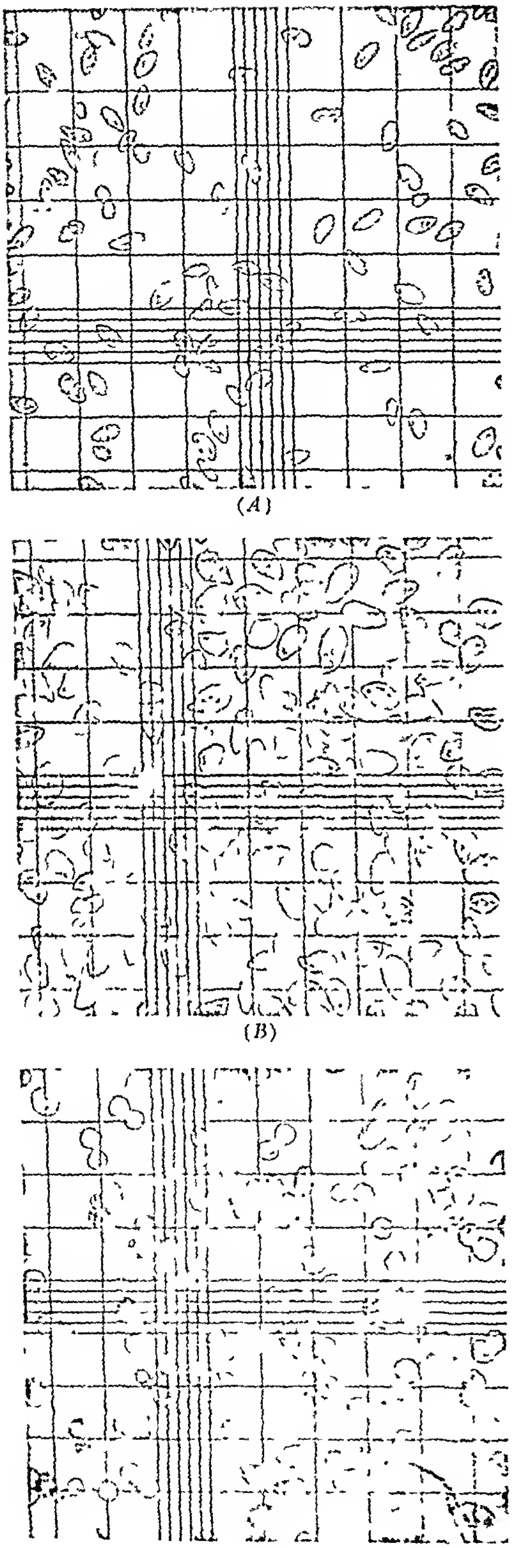

Fig 2—(A) Normal cells, (B) heat-treated cells, (C) synchronous dividing cells

The wiring diagram of this system is shown in Fig 1 The induced cell growth and synchronous cell division in *Tetrahymena pyriformis* is shown in Fig 2 The following is a brief account of its operation

The timer, a clock motor (Model 660, Automat Elec. Mfg Co) is used to close or open switch Sa at preset time intervals In the first half of the time cycle when switch Sa is on, coil Ca is energized and closes switches Sb and Sc. If the temperature is higher than 28 2°, thermostat (mercury thermoregulator, Aminco) Ta is in contact and energizes the coil in relay Ra to set on the compressor motor until the temperature is brought down just below 28 2° When the temperature is lower than 28°, thermostat Tb is open and coil Cb is energized to close switch Sd and the heating elements are on until the temperature reaches 28° From now on the room temperature (lower than 28°) and the heating unit operated by thermostat Tb, control the bath at 28° The compressor motor is used continuously and not intermittently for only twelve minutes in each period of one hour

In the second half of the time cycle when the timer opens switch Sa, thermostat Tc is in operation As shown in the diagram, Tc shares essentially the same circuit as Tb except that Tc is set at 34° In the present case, Tc is used to raise the temperature from 28° to 34° and maintains that temperature as long as switch Sa is in open position

To adjust the rates of heating and cooling between 28° and 34° according to the empirical findings of Scherbaum and Zeuthen (1), it is best to first adjust the quantity of water in the bath so that it cools from 34° to 28° in twelve minutes For warming up, one can easily estimate the wattage of the heating elements required so that the temperature can be raised to 34° from 28° in eight minutes

The surface area of our bath is 12 × 28 inches The amount of water used is about 30 L The compressor used is a Westinghouse Type E T-A Model and the heating elements are two copper-sheathed heaters of one kilowatt each

It will be more convenient if a time switch (Type 471, G E) is connected in the circuit so that it can set the timer switch on at midnight so that in the morning the heat-treated cells are ready for the studies on cell growth or cell division

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Studies on Cell Growth and Cell Division II*

Selective Activity of Chloramphenicol and Azaserine on Cell Growth and Cell Division

By KWAN-HUA LEE, YOKO OKANO YUZURIHA, and JOHN J. EILER

A simplified practical procedure for the study of drug action on cell growth and cell division as two distinct processes is described. To illustrate one of the many uses of this system, the selective activity of two antibiotics, chloramphenicol and azaserine on cell growth and cell division is demonstrated.

IN MASS CULTURES of unicellular organisms, cells grow and divide at random The conventional methods using mass cultures in the study of drug actions, have provided valid estimates of the overall biological activity of drugs, but have not permitted a clear cut differentiation between effects of drugs on cell growth and cell division

Recently, Scherbaum and Zeuthen (1) developed a controlled temperature shift technique which permits the study of cell growth and cell division as separate processes in mass culture of *Tetrahymena pyriformis* When a culture of *Tetrahymena pyriformis* GL is exposed to alternate half hour periods at 28° (optimum for growth) and 34° (sublethal) for seven or eight cycles, the cells continue to grow in size up to about three times the size of the initial normal cells, with little or no increase in cell number During this temperature-cycling period, it is possible to study the effect of drugs on cell growth alone When these overgrown heat-treated cells are maintained at 28°, either in a nutrient medium or in a nutrient free salt buffer solution (2), they go into cell division synchrony three times before normal random cell division is resumed In the nutrient medium, both cell division and cell growth take place, while in the buffer solution only cell division is observed During this cell-dividing period, it is possible to study the effect of drugs on cell division

In the first paper of this series (3), there was presented the design of an automatic temperature control system which duplicates nicely the conditions established by Scherbaum and Zeuthen (1) as being necessary to the separation of the two phases of cell regeneration

In this paper, a simplified practical procedure to test the effect of drugs on either the growth or the division process, or on both, in *Tetrahymena pyriformis* is presented

To illustrate one of the uses of this procedure, the results of the study of the selective activity of two antibiotics, chloramphenicol and azaserine,[1] are demonstrated Chloramphenicol, in appropriate concentrations, inhibits cell growth but not cell division, while azaserine inhibits cell division, but not cell growth

METHODS

Culture—*Tetrahymena pyriformis* GL was obtained through the courtesy of Professor G W Kidder and maintained in a proteose-peptone (Difco), liver extract fraction L (Wilson Laboratories) and salt medium (1) Stock cultures were transferred daily by means of an inoculating loop to 6 ml of medium in test tubes (15 × 150 mm) in a slanted position and maintained at 28° For the experimental culture, 200 ml of medium in a 500 ml Erlenmeyer flask was inoculated with 0 3 ml of a two day old culture (containing about 10^5 cells) and maintained at 28° At the end of eighteen hours, the culture contained about 35,000 cells/ml and was used in the heat treatment as described below

Cell Growth.—For the study of the effects of the drugs on cell growth, 20-ml portions of the experimental culture were transferred into 250 ml Erlenmeyer flasks Samples to serve as initial controls for cell count and protein content were taken at this time The drug under study was first dissolved in approximately 0 1 ml of pH 6 8 salt buffer solution (2) and then added to some of the flasks These flasks together with three controls were then clamped on the shaking device which has a horizontal movement of 4 cm displacement at fifty strokes per minute in the temperature controlled water bath (3) and subjected for seven cycles of heat treatment Each cycle consisted of half an hour at 34° followed by half an hour at 28° At the end of the seventh cycle, the growth of the cells in the flask was stopped by the addition of two drops of Bouin's formaldehyde reagent (4) or by immersing the flasks in a boiling water bath for not more than fifteen seconds Prolonged heating or too much formaldehyde reagent added caused difficulties in solubilization of the cells in biuret reagent The formaldehyde-treated or heat-killed cells were transferred to 50 ml conical centrifuge tubes and centrifuged The cells were washed twice with buffer solution and then analyzed for protein content by the use of the biuret method (5) When the amount of cell protein analyzed was less than 4 mg, removal of lipid by repeated extraction with alcohol-

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[1] Graciously supplied by Dr F L Wittle of Parke Davis Research Laboratories Detroit Mich

ether mixture (1:1 v/v) or trichloroacetic acid-alcohol mixture (6) is not necessary.

For the study of cell growth in the absence of drug, a series of flasks, each containing 20 ml. of experimental culture, were subjected to the temperature cycling as described above. Flasks were removed from the bath at the end of each cycle and at the end of the period of synchronous cell division, as indicated in Fig. 1, for cell protein analysis and cell counting.

Cell Division.—For cell division studies in the absence of nutrient, the cells in one of the three flasks used as controls in the growth studies, at the end of the last cycle, were washed with buffer solution in the hand centrifuge. The washed cells were then suspended in 10 ml. of the buffer and initial samples for cell count were taken. One-ml. portions of the cell suspension were transferred to a series of 50-ml. centrifuge tubes containing one ml. of buffer with the appropriate concentrations of drug and placed in the water bath at 28°. The time required for preparing these cells for the division studies was less than five minutes. After five and one-half hours, 0.6 ml. of formaldehyde reagent was added to each tube and the cells were counted in a Sedgwick-Rafter counting chamber, using a Whipple ocular micrometer disk (7).

For the studies on the effect of drug on cell division in the presence of nutrient, a series of flasks, each containing 20 ml. of experimental culture, were subjected to temperature cycling as described. At the end of the last cycle, samples were taken for cell count and the drug in appropriate concentrations was added to some of the flasks and the incubation was continued at 28°. At the end of five and one-half hours, samples were taken for cell count.

RESULTS

Cell Growth and Cell Division.—The results of the study of cell growth and cell division in the absence of drug are presented in Fig. 1. The ordinate expresses the changes in cell protein (growth) and cell count (division) relative to the initial values. The drawings in the figure are to indicate the approximate change in cell size and cell number during the various procedures. The average amount of protein in the initial log phase cell was about 3×10^{-3} μg./cell (with a variation from 2.6 to 3.2×10^{-3} μg./cell). The average initial cell count was 35,000 cells/ml.

In Fig. 1 it should be clear that during the period of temperature cycling, the increase in cell protein was not accompanied by any significant increase in cell number. During this period the protein content of the constant population of cells increased to more than three times that of the initial normal cells. Such an increase is in excellent agreement with other measures of the cell growth during this period, such as dry weights (8) and cell volume (9). It should also be noted that an increase in total protein took place during the period of constant temperature when cell division was permitted to occur in the *presence* of nutrient. Here, over a period of five and one-half hours the protein of the cells increased to double the value at the end of the temperature cycling and to six times that of the initial value. The increase in protein during the period of constant temperature was, of course, accompanied by cell

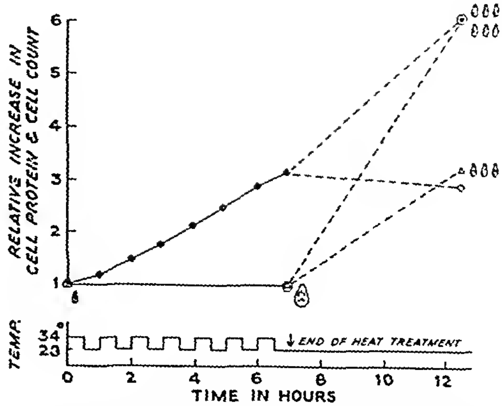

Fig. 1.—Relative change in cell protein and cell count under the various conditions in the absence of drug. The symbols Δ (relative cell count) and ◊ (relative protein) refer to those experiments wherein the cells were transferred to nonnutrient medium after the seven hours of heat treatment in the presence of nutrient. The symbols O (relative cell count) and ● (relative protein) refer to those experiments in which the cells were maintained in nutrient. The drawing indicates the increases in size and number of *Tetrahymena* under the several conditions.

division. Interestingly, simple calculations permit the view that during both periods the increases in protein were linearly related to the time spent at 28°, regardless of the occurrence or nonoccurrence of cell division. No increase in protein was observed when cell division was permitted to take place in the *absence* of nutrient. Indeed, there was observed a slight decrease in protein, probably due to catabolic utilization.

The increases in cell number during the period of constant temperature, both in the presence and in the absence of nutrient, were in good agreement with the increases in cell protein noted above. We have observed the three expected (1,2) sets of synchronous division both in the presence and in the absence of nutrient. In the absence of nutrient, during the five and one-half hours at constant temperature, the cell population increased to a value 3.2 times the starting population. Such an increase was made possible by the increase in protein which took place during heat treatment. In the presence of nutrient, there was an increase in cell population to six times the original. The increase in population was matched almost precisely by the increases in protein which took place during the two periods.

It should be clear from Fig. 1 that at the end of the period of cell division, both in the presence and in the absence of nutrient, the amount of protein per cell is practically the same as that of the initial log phase cell. Further, it should be evident that the protein content of the cell mass is a good index of cell growth during the period of temperature cycling, and that a single cell count at the end of five and one-half hours, in the absence of nutrient, is a sound measure of the incidence of cell division. These simple tests have been used to evaluate the actions of chloramphenicol and azaserine.

Effect of Chloramphenicol on Cell Growth and Cell Division.—In Fig. 2, the results of the effect of chloramphenicol on cell growth and cell division,

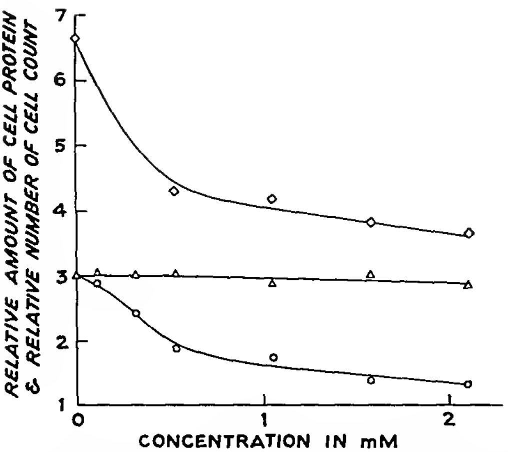

Fig. 2.—Effect of various concentrations (mM per liter) of chloramphenicol on cell protein and cell count. ○ = Relative amount of cell protein at the end of heat-treatment period in nutrient medium. ◇ = Relative cell count five and one-half hours after heat treatment in nutrient medium. △ = Relative cell count five and one-half hours after heat treatment in nutrient-free medium.

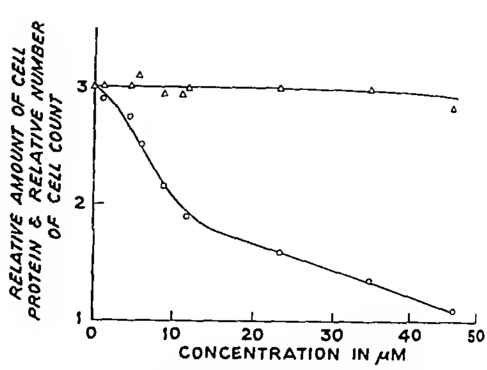

Fig. 3—Effect of various concentrations (μM per liter) of azaserine on cell protein and cell count. △ = Relative amount of cell protein at the end of heat-treatment period in nutrient medium. ○ = Relative cell count five and one-half hours after heat treatment in nutrient-free medium.

as measured by protein content and cell count, are expressed in relation to the initial values for each parameter. In the absence of chloramphenicol, the amount of cell protein increased during the period of heat treatment to three times the initial value. Under identical conditions, in the presence of graded concentrations of chloramphenicol, there is a definite inhibition on protein synthesis (Fig. 2). The extent of inhibition is directly related to the concentration of drug present.

In the presence of the same range of concentrations of chloramphenicol as used in the growth studies, cell division in the absence of nutrient is not influenced to any measurable extent (Fig. 2). However, when cell division is permitted to take place in the presence of nutrient, chloramphenicol is definitely inhibitory. Clearly, the inhibitory effect of the drug under these conditions is a consequence of its primary effect on cell growth.

Effect of Azaserine on Cell Growth and Cell Division.—The effect of azaserine on cell growth and cell division is shown in Fig. 3, which is plotted in the manner indicated for Fig. 2. In exact contrast to the action of chloramphenicol, azaserine, within the concentrations studied, has no effect on cell protein synthesis, but has a definite inhibitory action on cell division. At a concentration of 8 μg. per ml. (4×10^{-5} M), cell division is almost completely blocked. This same concentration of azaserine shows very little inhibition on cell growth as indicated in Fig. 3.

The addition of azaserine during the period of heat treatment has no observable effect on the subsequent cell division. In one set of experiments 2 μg. of azaserine was added to each ml. (1×10^{-5} M) of culture at the beginning of the period of heat treatment. At the end of heat treatment, the cells were washed with buffer and were observed to give the same number of new cells as did the control culture. The result strongly suggests that azaserine does not affect any process in the growth phase that is connected with subsequent cell division.

DISCUSSION

During the period of temperature cycling, the increase in cell protein observed in this study is almost identical to the increases in dry weight (8), cell volume (9), ribonucleic acid (8), and deoxyribonucleic acid (8) observed by others. This indicates that the growth which takes place during this period, in the absence of drugs, is balanced, in contrast to the unbalanced growth observed by Cohen and Barner (10) in a mutant strain of *E. coli*. The balanced nature of the growth is further supported by the fact that the number of cells that can be derived in nutrient-free medium are directly related to the extent of the increase in growth in the heat-treated cells. In the absence of added drug, any one of the above mentioned parameters may be used to measure cell growth. In the presence of drug, the balance of growth may be disturbed in several possible ways, depending upon the drug and other factors. However, in the present studies, cell protein appears to have served as a valid measure of growth in the presence of either of the two antibiotics.

In the absence of drug, five and one-half hours are required, either in the presence or absence of nutrient, for the completion of the three successive synchronous cell divisions. The difference in cell count in the presence and absence of drug in buffer medium at the end of five and one-half hours serves as a measure of the overall effect of the drug on cell division.

The inhibitory effect of chloramphenicol has been studied in other laboratories (11, 12). The results of the present study indicate that chloramphenicol inhibits the growth of *Tetrahymena pyriformis* under conditions in which cell division does not take place. The results provide experimental evidence that chloramphenicol does not affect cell division at concentrations in which cell growth is inhibited to a great extent. Indeed, it is clear that chloramphenicol inhibits cell division only when that division takes place in nutrient accompanied by cell growth. Clearly, the inhibition of division is a consequence of the inhibition of growth.

The inhibitory action of azaserine on cell regeneration has been reported in a large number of unicellular organisms (13). This is the first time, to our knowledge, its effect has been studied on

Tetrahymena pyriformis In *Tetrahymena pyriformis*, azaserine definitely inhibits cell division at concentrations which have little or no effect on growth. Its effects on cell division cannot take place during the period of cell growth Two points which concern the probable mode of action of azaserine are worthy of note *Tetrahymena pyriformis* requires preformed purines and pyrimidines and cannot synthesize either from precursors (14) In our studies, it has been shown that azaserine inhibits the features of cell division which take place in a nutrient-free medium, after the necessary ribonucleic acid and deoxyribonucleic acid have been accumulated. Accordingly, in these organisms, the drug cannot exert its effect through inhibition of purine synthesis as has been proposed (15)

Studies on the more detailed mechanism of action of these drugs are in progress

SUMMARY

The controlled-temperature shift technique of Scherbaum and Zeuthen for inducing cell growth and cell division as two distinct processes in mass cultures of *Tetrahymena pyriformis* GL has been restudied. A simplified practical method for measuring cell growth and cell division and the action of drug thereon has been described.

Using this method, the selective activity of two antibiotics, chloramphenicol and azaserine, has been tested. Chloramphenicol, in appropriate concentrations, inhibits protein synthesis, but not cell division; while azaserine, even at a concentration almost completely inhibiting cell division, has very little effect on cell growth.

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Sulfaethylthiadiazole V*

Design and Study of an Oral Sustained Release Dosage Form

By MANFORD J. ROBINSON and JOSEPH V. SWINTOSKY

An aqueous sustained release suspension of sulfaethidole (sulfaethylthiadiazole or SETD) was prepared. The velocity constant (k_t) for drug disappearance from blood in adult human subjects was utilized to approximate the quantity of drug to be administered every twelve hours in order to maintain free SETD blood concentrations near 10 mg. per cent. Dissolution characteristics of this sustained release suspension were also studied *in vitro* in three different test fluids. Possible relationships with the foregoing *in vivo* results are discussed.

THIS PAPER outlines and discusses some of the methods we have utilized in the design, preparation, and evaluation of a liquid sustained release dosage form

Blythe (1) has discussed general concepts relating to the formulation and evaluation of sustained release products. He describes oral sustained release dosage forms as preparations which provide a sustained therapeutic effect by first releasing a therapeutic dose, then gradually and continually releasing additional medication over a prolonged period Nelson (2) has discussed the estimation of amounts of drug needed to meet the requirements for sustained release. In previous publications (3–7) we have shown that orally administered sulfaethidole (sulfaethylthiadiazole or SETD) is eliminated from blood at approximately a first-order rate for a considerable period of time following attainment of "pseudo-steady state" diffusion equilibrium Applications of the use of k_b, the velocity constant for drug elimination from blood, in the design and study of sustained release preparations have been discussed and illustrated (5, 6, 8) Methods for determining rate of dissolution *in vitro* for sustained release products

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have been presented (9–11), although these methods have been designed primarily for control purposes. We have also discussed (8) the possible interrelationships of *in vitro* dissolution rates, blood concentrations, and urinary excretion for two aqueous suspensions of coated sulfamethylthiadiazole.

Recently, an aqueous sustained release preparation of SETD was developed in our laboratories. This drug has been extensively investigated in Europe and found to be a safe, clinically effective sulfonamide (12–15). Recent studies in the United States (16–18) have confirmed the European findings. Frisk (19) has determined blood and urinary concentrations in subjects receiving oral and intravenous doses of SETD. Our investigation of the kinetics of absorption, distribution, and excretion of this drug has been reported (5). Although this drug is normally administered in large doses, the rapidity with which SETD is absorbed and its relatively slower excretion indicated that it could be formulated into a preparation for oral administration every twelve hours. A sustained release form of SETD, by prolonging absorption, should provide relatively uniform, therapeutically effective tissue concentrations for the twelve-hour time period, and also decrease peak loads on the kidney during the early time periods following oral administration. SETD is particularly applicable to this type of study, since it can be readily analyzed in blood and urine and there is a correlation between blood concentration and therapeutic efficacy.

EXPERIMENTAL

Preparation of the Dosage Form.—The sustained release powder was prepared by mixing SETD (40% w/w) with molten hydrogenated castor oil maintained at 110°. The suspension was then spray congealed, using a centrifugal wheel atomizer, to yield a powder having an average surface volume diameter (Fisher Subsieve analysis) of 35 μ. The average surface volume diameter of the SETD used in preparing the sustained release powder was less than 4.0 μ. The sustained release powder was suspended in an aqueous vehicle at a concentration equivalent to 3.9 Gm. of SETD per 30 ml.

In Vitro Dissolution Studies.—The amounts of SETD released in various time intervals for the suspension were determined in three test fluids. Formulas for these fluids and amounts of suspension added in SETD equivalents are shown in Table 1. The methods for measuring SETD dissolution in fluids A and B were those previously described for sulfamethylthiadiazole (8).

Test fluid C was developed for control purposes. This fluid was prepared by dissolving the buffer salts in distilled water, adjusting the pH to 6.4 ± 0.03 and, immediately prior to use, adding ox bile extract and pancreatin. Samples of the sustained release suspension equivalent to 0.125 Gm. of SETD were

TABLE I.—COMPOSITION OF IN VITRO TEST FLUIDS AND AMOUNTS OF SUSTAINED RELEASE SUSPENSION ADDED IN SETD EQUIVALENT

Test Fluid	pH	Composition	SETD Content
A	1.2	0.1 *N* HCL	2 mg. per cenr
B	8 3	Ox Bile Extract—2.0 Gm., Pancreatin—0.56 Gm. Sodium Bicarbonate—3.0 Gm., Distilled water sufficient to yield 200 ml.	10 mg. per ml.
C	6 4	Citric Acid, hydrous—6.46 Gm., Na_2HPO_4, anhydrous—19.76 Gm., Distilled Water sufficient to yield 1,000 ml. Ox Bile Extract—1.0 Gm., Pancreatin 0.28 Gm. were added to 100 ml. of the buffer solution.	0.125 Gm. per 60 ml.

added to 60 ml. of the control fluid in 90-ml. screw-capped containers. These were placed in a constant temperature bath at 30° ± 0.1 and rotated at 40 r. p. m. Samples were removed after one-fourth, one, three, and six hours, filtered, and the residue assayed for SETD content. SETD was extracted from the residue using boiling 1.0 *N* HCl and determined by potentiometric titration with standardized 0.05 *M* $NaNO_2$.

Blood and Urine Concentration Studies.—The subjects receiving oral doses of the sustained release suspension were healthy, ambulatory adults. The initial dose was administered to each subject immediately before breakfast. Food and fluid ingestion was then permitted *ad libitum*. Blood samples were withdrawn by venipuncture of the antecubital vein. Complete urine collections were made for measurement of total drug output. Dosage regimens and blood and urine sample collection time for these studies were as follows: *Part A.*—12 subjects received single oral doses equivalent to 3.9 Gm. of SETD. Blood samples were withdrawn at two, four, six, eight, ten, and twelve hours. *Part B.*—10 subjects received oral doses equivalent to 3.9 Gm. of SETD initially, followed by 1.95 Gm. every twelve hours for five consecutive doses, totaling approximately 13.65 Gm. Blood samples were taken periodically for seventy-two hours. Complete urine collections were made at twelve-hour intervals for ninety-six hours.

The concentrations of free and free plus conjugated (total) SETD in the blood and urine were determined by previously described procedures (4, 5).

A single lot of the sustained release suspension (X46) was used for the aforementioned studies. To illustrate duration of absorption we have included other unpublished blood concentration and urinary excretion data which were obtained during the development of this product. The data are taken from 144 tests in which single doses of the sustained release suspension were administered orally to 61 adult subjects, and from 5 tests in which 23 adult subjects received single 3.9-Gm. oral doses of *crystalline* SETD. The procedure of part A was followed except that in the case of the sustained release suspension an additional blood sample was taken at twenty-four hours. Complete urine collections were *made*

for four, eight, twelve, twenty-four, forty-eight, and seventy-two hours for both the sustained release suspension and crystalline SETD. The lots of the sustained released suspension were of the same composition as lot X46, had approximately the same particle size, and exhibited similar dissolution in the control test fluid (C). The crystalline SETD was administered either in capsules or as an aqueous suspension.

RESULTS AND DISCUSSION

In Vitro Dissolution Studies.—The amounts dissolved and periods for dissolution of the sustained release suspension in test fluids A, B, and C are shown in Table II. Approximately 24% of the SETD was found to dissolve in solution A after fifteen minutes and 41% after one hour. Dissolution in solution B is rapid and can be considered essentially complete (90%) in three hours. Approximately 80% of the SETD is released in test solution C after six hours. Differences in the amount of dissolution in each of the fluids appear to be minimal at the fifteen-minute period. The rate of drug release is not linear and decreases with time after the fifteen-minute period.

TABLE II.—DISSOLUTION OF SETD IN TEST FLUIDS A, B, and C

Time, Hr.	Dissolution, %[a] A	B	C
1/4	24	38	32
1	41	76	60
2	49		.
3	.	90	74
6	..	95	81

[a] Average of two or more determinations.

The powder used in preparing lot X46 was examined microscopically in a mineral oil mull and also allowed to disintegrate while being observed under a microscope using chloroform as a solvent for the hydrogenated castor oil. The individual spheres consisted of finely divided SETD particles uniformly dispersed throughout a matrix of hydrogenated castor oil. There was a thin surface film of hydrogenated castor oil distributed uniformly over the surface of each sphere. The observed similarity of initial release and the nonlinear decrease in dissolution rate with time are as would be anticipated for a product having the above physical composition.

Since drug dissolution rate should directly influence absorption, the lack of linearity observed in the *in vitro* tests should be reflected in the observed drug concentration in blood and excretion in urine. As the rate of dissolution in the gastrointestinal tract falls below that necessary to maintain the desired absorption rate a corresponding decrease in blood and urine SETD content would be expected.

Estimate of Drug Dose.—Previous publications (2, 4, 5, 6, 8) have outlined methods for estimating the amount of drug required to maintain a given blood concentration for any time interval. For drugs such as SETD, which are rapidly and completely absorbed and are not significantly degraded or biotransformed, the total sustained release dose may be estimated using the equation, $A = k_b ch + c$, where A equals the total drug dose, k_b is the rate of drug disappearance from blood following attainment of "pseudo-steady state" diffusion equilibrium and essentially complete absorption, c is the amount of drug required to obtain the desired blood concentration, and h is the number of hours that the selected blood concentration is to be maintained following attainment of "pseudo-steady state" diffusion equilibrium and essentially complete absorption. When estimating initial and subsequent sustained release doses for multiple dosage regimens the initial dose is approximately equal to A and subsequent doses A' are approximately equal to $k_b ch$.

We have shown (3–6) that a 2.0-Gm. dose of SETD provides a blood concentration near 10 mg. %, that "psuedo-steady state" diffusion equilibrium is attained, and absorption is essentially complete after about two hours, and that the k_b for SETD disappearance[1] from blood is in the order of .07 to .12. We have shown also (6) that an initial dose of 2.0 Gm. of SETD followed by 0.22 Gm. every hour will provide and maintain free blood concentrations near 10 mg. %.

Estimation of SETD dosage is complicated by a number of factors which are not readily amenable to calculation. It is desirable to limit the dosage form to a single package or unit. Since subsequent as well as initial doses of the sustained release suspension will contain nearly 50% of SETD available for rapid absorption, the blood levels must be allowed to subside somewhat in the later hours prior to administration of subsequent doses. It has been proposed that this occur at ten hours. However, for products which exhibit nonlinear type dissolution and for drugs which are rapidly absorbed and relatively slowly excreted (5) the blood concentrations possibly should subside at an earlier time. Further, the initial and subsequent twelve-hour doses must be such that they can be prescribed in units which are readily measured by the consumer. The consumer may be either an adult receiving a fixed amount of drug or an infant or child receiving the product on a mg./Kg. or gr./lb. basis.

An SETD dosage regimen of 3.9 Gm. for the initial twelve-hour period followed by one-half of the original dose (1.95 Gm.) every twelve hours is compatible with the above factors and is in reasonable accord with the calculated estimate. This dosage regimen should provide blood concentrations near the desired 10 mg. % for the twelve-hour time interval. The 3.9-Gm. SETD dose corresponds to an initial dose near 2 Gm. to hasten the approach to the steady state and approximately 1.9 Gm. to maintain this blood concentration.

Blood and Urine Concentration Studies.—*Part A.*—The individual blood concentrations observed for 12 subjects receiving a single dose of the sustained release suspension (X46) equivalent to 3.9 Gm. of SETD are shown in Table III. The minimum, maximum, and average free SETD blood concentrations for these subjects are shown in Fig. 1. The mean blood concentrations are near 10 mg. % for the observed ten hours and are in reasonable correspondence with those found for similar subjects receiving an initial 2.0-Gm. dose of SETD followed by 0.22-Gm. doses of SETD every hour (6). This is further illustrated in Table IV for the only subject (Ja) receiving both dosage regimens. The correspondence in blood concentrations indicates that the

[1] k_b here is .07 to .12 hour^{-1}, or may be expressed as 7 to 12% per hour.

sustained release suspension is providing an initial release near 50% of SETD and that the balance is being absorbed over most of the observed time period.

The average total excretion in urine four hours after oral administration of crystalline SETD in 1.0, 2.0, and 3.0 Gm. doses previously observed for 4 subjects was 200, 460, and 775 mg., respectively. The average total excretion in urine for subjects receiving single doses of the sustained release suspension (X46) was 466 mg. after four hours. The close correspondence in the four hour excretion values for a 2.0 Gm. dose of crystalline SETD and a single dose of the sustained release suspension also indicates that approximately 50% of the drug content is available for early absorption from the suspension.

Average free blood concentrations over a twenty-four hour period for 61 subjects receiving a single dose of the sustained release product equivalent to 3.9 Gm. of SETD are shown in Fig. 2. Cumulative drug excretion in urine for 49 of these subjects over seventy-two hours is shown in Table V. These data are in reasonable agreement with those observed for lot X46. It has been reported previously (4, 8) that absorption may be considered essentially terminated when the slope of the first order rate equation plots of drug disappearance from blood becomes identical for the dosage forms studied. For this product the semilogarithmic plot of the average free SETD blood concentration approximates a straight line after six hours. The slope of this line, however, indicates a rate of disappearance (k_b) near .05 hour^{-1} rather than .07 to .12 hour^{-1} observed for crystalline SETD. This difference in SETD excretion is also illustrated in Table V which compares the average cumulative drug excretion in urine over a seventy-two hour period for 23 adult subjects receiving crystalline SETD with that for 49 adult subjects receiving the sustained release product. These data indicate that absorption is apparently occurring after six hours but at a rate which with time becomes increasingly less than that required to maintain a constant blood concentration.

The indication that approximately 2.0 Gm. is available for early absorption and the prolonged release which may be inferred from the extended absorption time agree generally, but do not correlate precisely, with the results which were anticipated from the *in vitro* dissolution study.

Part B.—Clinical studies have shown the therapeutic efficacy and safety of SETD when administered according to a dosage regimen of 1.0 Gm. every six hours (16). It has been shown (6) that in the type of subject being studied such a regimen will provide steady state total SETD blood concentrations lying between 6.5 and 18 mg. %. The sustained release product should provide comparable

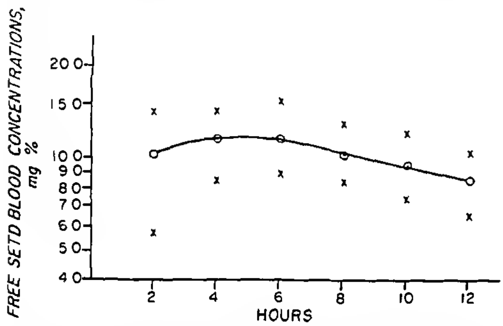

Fig. 1.—Minimum, maximum, and average free SETD blood concentrations for 12 adult human subjects receiving a single oral dose of the sustained release suspension X46 equivalent to 3.9 Gm. of SETD.

TABLE III.—FREE BLOOD CONCENTRATIONS (mg. %) FOR ADULT HUMAN SUBJECTS RECEIVING A SINGLE ORAL DOSE OF SUSTAINED RELEASE SUSPENSION (X46) EQUIVALENT TO 3.9 GM. OF SETD

Subject	Hours 2	4	6	8	10	12
Br	14.3	14.2	12.8	10.9	9.6	8.5
Ca	11.6	14.0	15.4	13.0	12.1	10.5
Cr	11.6	10.3	8.9	8.4	7.9	7.5
Ha	11.3	12.2	9.8	8.4	7.4	7.2
Jo	5.7	8.5	10.5	9.0	8.8	8.4
Bo	6.7	10.1	10.8	9.5	9.4	9.0
Ra	9.8	10.1	10.8	11.0	10.3	9.4
Ch	12.5	12.3	12.0	10.5	10.3	8.4
Mi	10.8	11.4	11.6	10.3	9.8	8.8
In	10.5	14.0	13.0	11.8	10.3	9.4
Cl	9.8	10.5	10.8	9.8	9.4	8.5
Je	10.1	10.3	11.4	10.8	8.1	6.5

TABLE V.—AVERAGE SETD EXCRETION IN URINE OVER A SEVENTY-TWO HOUR PERIOD FOR 49 ADULT SUBJECTS RECEIVING A SINGLE ORAL DOSE OF THE SUSTAINED RELEASE PRODUCT EQUIVALENT TO 3.9 GM. OF SETD AND 23 ADULT SUBJECTS RECEIVING ORAL DOSES OF APPROXIMATELY 3.9 GM. OF CRYSTALLINE SETD

Collection Time	Average SETD Excretion in Urine, mg. SETD	Sustained Release Product
0–4	1149	451
4–8	821	720
8–12	407	473
12–24	608	714
24–48	472	648
48–72	106	202

TABLE IV.—FREE AND TOTAL BLOOD CONCENTRATIONS FOR SUBJECT JA FOLLOWING A SINGLE ORAL DOSE OF THE SUSTAINED RELEASE SUSPENSION (X46) EQUIVALENT TO 3.9 GM. OF SETD AND ORAL DOSES OF 2.0 GM. FOLLOWED BY 0.22 GM. EVERY HOUR FOR EIGHT HOURS OF SETD

Material	Hours 1	2	4	6	8	10	12
SETD capsules							
Total	9.9	13.2	13.4	12.3	11.2		9.3
Free	9.5	12.8	12.3	11.8	11.0		8.7
Sustained release (X46) suspension							
Total		9.0	11.5	12.7	11.1	10.2	9.3
Free		8.8	10.9	11.5	9.7	9.4	8.6

blood concentrations when a dose approximating 2.0 Gm. of SETD is administered every twelve hours. The observed total SETD blood concentrations for 10 subjects receiving oral doses of the sustained release suspension (X46) equivalent to 3.9 Gm. of SETD initially followed by 1.95 Gm. of SETD every twelve hours for 5 consecutive doses are shown in Fig. 3 (the free drug concentrations were only slightly lower, as has been noted previously (6)). Circles denoting the average concentration in blood have generally been connected by lines. These lines do not necessarily indicate the precise concentration at any time but were added to aid in visualization. The vertical distances between the starred points denote the limits of observed total drug concentration in blood. The mean experimental values shown in Fig. 3 are reasonably constant throughout the entire period, and the concentrations generally are within the theoretical limits calculated for a dosage regimen of 1.0 Gm. of crystalline SETD administered every six hours. The blood concentrations also correspond closely to those previously reported (6) for similar subjects receiving 2.0 Gm. of crystalline SETD followed by 1.0-Gm. dose every six hours for 11 consecutive doses. For purposes of comparison these data are shown in Fig. 4

Figure 5 illustrates the constant twelve-hour excretion pattern of SETD following establishment of diffusion equilibrium and steady state blood concentration. Absorption efficiency may be calculated from the slope of the line during its straight line phase. The theoretical slope for this dosage regimen, when absorption and urinary excretion efficiency are 100%, is 0.163 Gm./hour The slope calculated from Fig. 5 is 0.16 Gm./hour, indicating an average absorption efficiency greater than 95%, for these subjects. Total SETD recoveries in urine after ninety-six hours for the subjects receiving the dosage regimens shown in Figs. 3 and 5 ranged from 87–100% with an average of approximately 93%. An average of 6.5% of the drug found in urine was biotransformed. This amount of biotransformation, based on urinary data, compares favorably with the 11% previously reported (6) for similar subjects receiving multiple oral doses of crystalline SETD

These data for multiple doses of the sustained release suspension indicate that when SETD is incorporated in sustained release form for twice daily oral dosage the physician may expect to obtain blood concentrations at least equivalent to those previously obtained on a four times daily regimen Further, the prolongation of absorption time through sustained release does not decrease the efficiency of total absorption nor increase the per cent biotransformation of this drug.

These experimental data in conjunction with previously reported data (3, 4, 5, 6, 8) illustrate some of

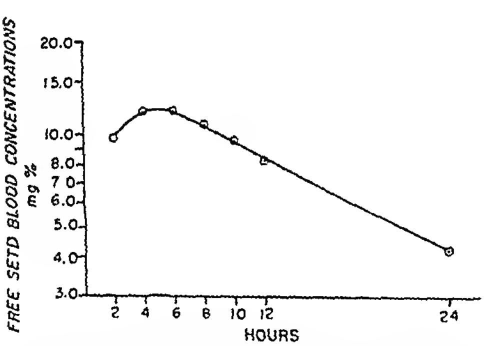

Fig. 2.—Average free SETD blood concentrations for 61 adult human subjects receiving a single oral dose of the sustained release product equivalent to 3.9 Gm. of SETD.

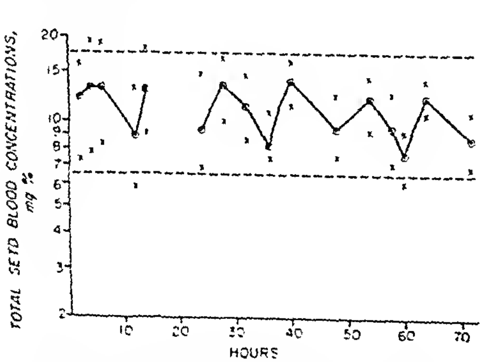

Fig. 3.—Total SETD blood concentration data for 10 adult human subjects receiving the sustained release suspension X46 in doses equivalent to 1.95 Gm. every twelve hours. Broken lines indicate calculated maximum and minimum limits.

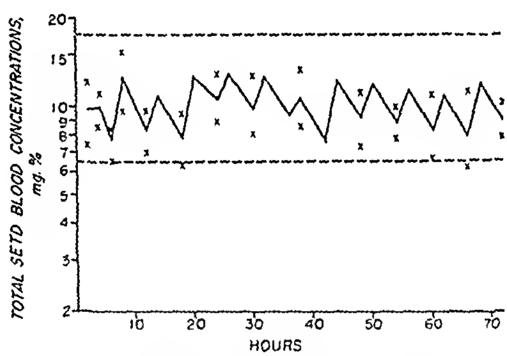

Fig. 4.—Total SETD blood concentration data for 4 adult human subjects (6) receiving 2 Gm SETD followed every six hours by a 1-Gm. dose for seventy-two hours. Broken lines indicate minimum and maximum calculated limits where the k_b is estimated to vary between .07–.12 hour^{-1} and the time lag for absorption T_l is estimated to be 2.0 hours.

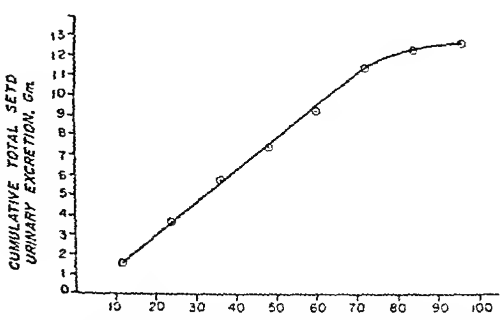

Fig. 5.—Average cumulative urinary excretion of total SETD for 10 adult subjects corresponding to the dosage regimen shown in Fig. 3.

the methods we have utilized for the design and evaluation of a sustained release dosage form The utility of the specific velocity constant for drug elimination from blood for estimating amounts of drug to be incorporated into and approximating the amount and duration of *in vivo* release for sustained release dosage forms is discussed The general relationships between results anticipated by *in vitro* tests and those observed *in vivo*, although not precise, may aid in the formulation of oral sustained release dosage forms

This approach should not only facilitate sustained release dosage form study and design, but may also aid in establishing standards of performance for some sustained release products

SUMMARY

1. A sustained release suspension of sulfaethylthiadiazole (SETD) was prepared by spray congealing a mixture of SETD (40 per cent w/w) in molten hydrogenated castor oil, and suspending the resulting powder in an aqueous vehicle.

2. The spray-congealed powder consisted of spherical particles having an average particle size of approximately 35 μ. Fine particles of SETD were uniformly distributed within the spheres.

3. Dissolution characteristics of the powder were determined under standardized conditions using three different *in vitro* test fluids. Differences in amounts of drug dissolved in each of the test fluids were minimal at fifteen minutes. The rate of release was not linear and decreased with time. Possible relationships between *in vitro* dissolution characteristics, the observed drug concentrations in blood, and excretion in urine for subjects receiving oral doses of the product are presented and discussed.

4. Single oral doses of the sustained release product equivalent to 3.9 Gm. of SETD, when administered to 12 adult human subjects, maintained blood concentrations near 10 mg. per cent during a two to twelve-hour period. Maximum concentrations occurred near six hours and subsided to the minimum at twelve hours.

5. Blood concentrations after two hours and the amount of drug collected in urine after four hours are very similar for subjects receiving orally either 3.9 Gm. of SETD in the sustained release suspension or 2.0 Gm. of uncoated crystalline SETD. This indicates that approximately 50 per cent of the sustained release dosage form is available for early absorption.

6. Although the greater part of the single oral dose was absorbed during the first six hours the remainder was apparently absorbed over an extended time interval, as evidenced by the slope of the blood concentration curve and drug excretion in urine. The apparent mean k_b from the slope of the straight line after six hours for the subjects studied was approximately .05 hour^{-1}, whereas for similar subjects receiving nonsustained crystalline SETD the actual k_t value ranged from .07 to .12 hour^{-1}. The amount of SETD excreted in urine over seventy-two hours for subjects receiving the sustained release product was found to be less in early and more in later time periods than for similar subjects receiving crystalline SETD.

7. Administration of multiple oral doses of the sustained release suspension equivalent to 3.9 Gm. of SETD for the first twelve hours followed by 1.95 Gm. every twelve hours resulted in blood concentration maxima and minima which correspond to those calculated and observed for a regimen of 2.0 Gm. of uncoated crystalline SETD for the first six hours followed by 1.0 Gm. every six hours.

8. Drug recovery from urine was used to determine minimum absorption efficiency under the conditions of this study. About 95 per cent of the administered dose was recovered in urine, an amount corresponding to that observed when crystalline SETD is administered.

9. Biotransformed SETD in urine averaged 6.5 per cent following administration of the sustained release suspension. This was of the same order of magnitude as observed for subjects receiving crystalline SETD.

10. Employment of the concepts embodying measurement of drug disappearance from blood and excretion in urine may facilitate study and design of sustained release dosage forms and be of value in establishing standards of performance for products where these values can be related to therapeutic response.

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Spectrophotofluorometry of Reserpine, Other Rauwolfia Alkaloids, and Related Compounds*

By R. P. HAYCOCK, P. B. SHETH, and W. J. MADER

The excitation and fluorescent characteristics of a number of Rauwolfia alkaloids and related compounds are reported using a Spectrophotofluorometer. A procedure for the determination of reserpine in mixtures of reserpine and rescinnamine is described.

THE PHENOMENA of natural fluorescence of reserpine and other rauwolfia alkaloids has been noted by various investigators since the isolation of reserpine was reported, and several investigators have utilized fluorescences for the determination of reserpine in dosage form and in biological tissues (1–3). However, these investigators employed filter type fluorometers which restrict the fluorescent detection to the visible region of the spectrum and to those fluorophors that absorb light corresponding to one of the available lines of the mercury emission spectrum. The development of spectrophotofluorometers has extended the scope of fluorescent analysis to permit the excitation of compounds and the measurement of resulting fluorescence throughout the ultraviolet and visible regions.

To date, little data have been reported on scanning spectrophotofluorometric measurements of rauwolfia alkaloids. This led to a survey of the excitation and fluorescent characteristics of a number of rauwolfia alkaloids and related compounds for possible usefulness as a basis for the identification and quantitative assay of reserpine in the presences of other rauwolfia alkaloids.

EXPERIMENTAL

Apparatus.—Aminco-Bowman Spectrophotofluorometer with silica cells, 1-cm. cross section, four transparent sides. Recorded spectra were obtained, using a Mosely Model No. 3 Flat Bed X-Y Recorder, modified for use with the Spectrophotofluorometer.

Solutions.—Dissolve 25.0 mg. of the compound in 1 ml. of chloroform and dilute to 100 ml with methanol. Dilute 3.0 ml. to exactly 50 ml. with methanol and further dilute 10 ml. to exactly 50 ml. with methanol. The diluted solution contains 3.0 μg of alkaloid per ml. Indole, norharmane, and harmaline are prepared to contain 1.5 μg per ml.

Technique.—The Aminco-Bowman Spectrophotofluorometer used in this study was calibrated using 0.1 μg. U. S. P. quinine sulfate per ml. in 0.1 *N* sulfuric acid in accordance with the technique of Sprince and Rowley (4). The excitation maximum was observed at 350 mμ, and the corresponding maximum fluorescent peak occurred at 450 mμ. The peaks checked within 5 mμ on repeated tests and agreed with the corrected values reported by Sprince and associate.

The fluorescent spectrum for each solution was scanned on the oscilloscope screen, while changing the excitation wavelength until the appearance of a fluorescent band. At the fluorescent maximum, the wavelength of maximum excitation was determined by manipulation of the excitation monochromator. Under these conditions, excitation and fluorescent maxima were obtained for each compound tested, as shown in Table I, together with the structural formula and relative fluorescent sensitivity. The relative fluorescent sensitivity is the product of the meter reading on the transmission scale and meter multiplication factor. Excitation and fluorescent spectra of reserpine, rescinnamine, 3-dehydroreserpine, and tetrahydroreserpine are presented in Figs. 1–4. These spectra are typical of the other alkaloids and related compounds.

METHOD DEVELOPMENT

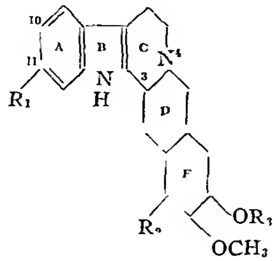
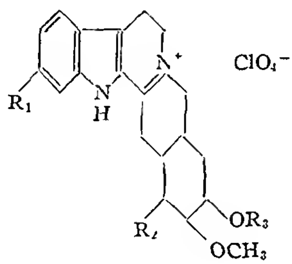
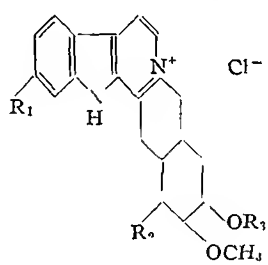
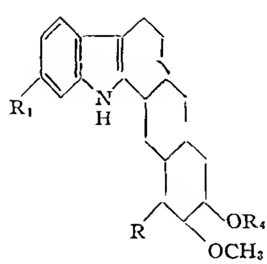
An adaptation of the Szalkowski-Mader colorimetric reaction of reserpine with nitrous acid is the official (5) and preferred procedure for determining reserpine in dosage form. In a prior publication (6) from this laboratory, it was established that the 7-methoxy-β-carboline group, i.e., the 11-methoxy group and the AB and C ring skeleton of reserpine, is the functional group responsible for the yellow pigment which is formed. Of the alkaloids containing this similarity to reserpine, methyl reserpate and reserpic acid are eliminated in the extraction procedure and dehydroreserpine and tetradehydroreserpine do not interfere. The other principal alkaloid found in various species of rauwolfia which contains this common structural similarity to reserpine and reacts with nitrous acid is rescinnamine. A preliminary examination of the fluorescent spectra of reserpine and rescinnamine showed that they exhibit different excitation and fluorescent spectra and led to a study of the practicability of determining reserpine in the presence of rescinnamine by spectrophotofluorometry.

Procedure.—A solution of reserpine in methanol was prepared as outlined under Experimental to give a range of 1 μg. to 5 μg. per ml. The excitation monochromator and fluorescent monochromator were set at 280 mμ and 360 mμ, respectively, at the maximal excitation and fluorescent response of reserpine. The relationship between concentration of reserpine and fluorescence is shown in Fig. 5. All measurements were made using $^1/_{16}$ inch defining slit (band pass = 6 mμ) and a 1P21 photomultiplier. The blank reading served as the fiducial point, and the instrument was set at an arbitrary point with the highest standard. The fluorescence is proportional to concentration in the range of 1 to 5 μg. per ml.

The above method has been successfully applied to mixtures of reserpine containing as much as 80

* Received February 26, 1959, from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.

TABLE I.—SENSITIVITY AND MAXIMUM EXCITATION AND FLUORESCENT WAVELENGTHS FOR SOME RAUWOLFIA ALKALOIDS AND RELATED INDOLES[a]

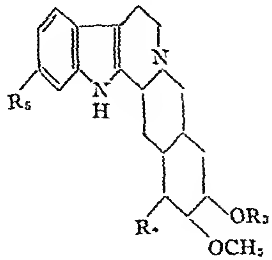
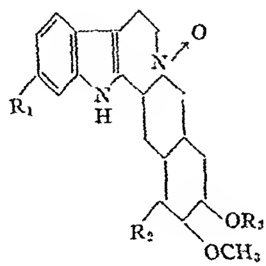
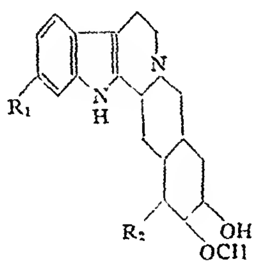
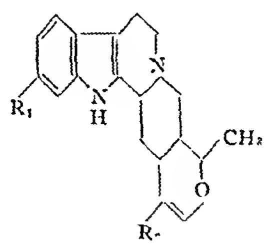
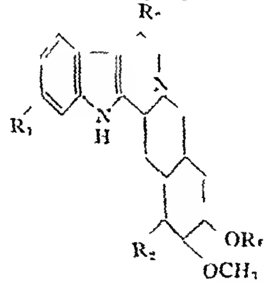
Compound	Structure	Wavelength mμ Excitation max	Wavelength mμ Fluorescence max	Relative Fluorescent Sensitivity
Reserpine		280	360	6
Isoreserpine		390	510	18
Dehydroreserpine perchlorate		390	510	75
Tetradehydroreserpine chloride		340	440	50
Rescinnamine		310	440	2

rescinnamine with recoveries within 2 to 3% of the theoretical reserpine content as shown in Table II. The reproducibility of replicate determinations was within 3%. This difference is not more than can be accounted for by analytical errors.

DISCUSSION

The following discussion is not meant to be a complete evaluation of the findings, rather it is intended only to point out the more evident results to emerge from the investigation. The excitation and fluorescent spectra of reserpine, reserpinine, deserpidine, renoxidine (reserpine N oxide), methyl reserpate, methyl O (3,5 dimethoxy-4 hydroxybenzoyl) reserpate, syrosingopine, and syrosingopine N oxide generally showed peaks at approximately 280 mμ with a corresponding fluorescent maximum at approximately 360 mμ. The order of magnitude of fluorescence depends upon certain groups in various positions of the basic alkaloid structure. For example, the 11 methoxy moiety impedes fluorescences as is evident by a comparison of reserpine with deserpidine, which does not possess an 11 methoxy group and produces an increase in fluorescences equivalent to 2½ above that of reserpine. Reserpinine, a tertiary indole alkaloid with a heterocyclic ring E and possessing an 11 methoxy substituent emits fluorescent light below that of reserpine. The introduction of oxygen at position 4, however, is an enhancing group as is evident by an increase in the fluorescence emission

TABLE I—*Continued*

Compound	Structure	Wavelength, mμ Excitation max	Fluorescence max	Relative Fluorescent Sensitivity
Deserpidine		280	360	15
Renoxidine (reserpine N-oxide)		280	360	8
Methyl reserpate		300	360	19
Reserpinine		300	360	2
Methyl O (3,5-dimethoxy-4 hydroxybenzoyl) reserpate		300	360	8

of renoxidine (reserpine N-oxide) and syrosingopine N-oxide. The esterified alcoholic function of reserpine, i. e., trimethoxybenzoic acid decreases the fluorescences considerably as can be seen by a comparison of the fluorescent emission of reserpine with methyl reserpate. The substitution of a hydroxyl group in the esterified function, i. e., 3,5-dimethoxy-4-hydroxybenzoic acid has an enhancing effect upon the fluorescence displayed by reserpine as is evident by the increased fluorescences of methyl O-(-3,5 dimethoxy-4-hydroxybenzoyl) *reserpate*.

The esterified alcoholic function of rescinnamine, i. e., trimethoxycinnamic acid, *is responsible for* a shift in the excitation maximum to 310 mμ and a corresponding shift in the fluorescent emission to 440 mμ. In addition to the shift, the fluorescent response is considerably weaker than that produced by the reserpine nucleus. This qualitative and quanti-

TABLE I—*Continued*

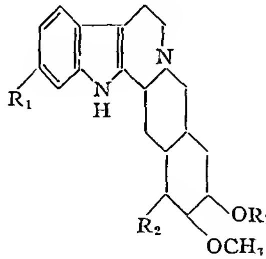
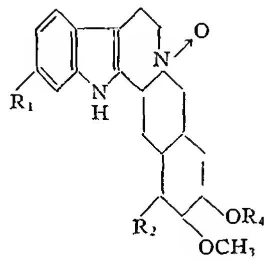
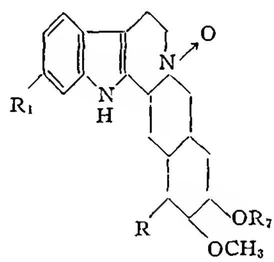
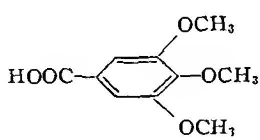
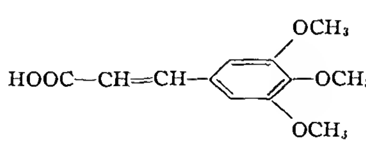
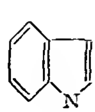
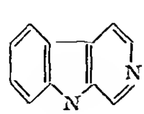
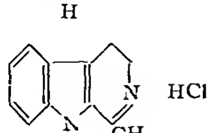
Compound	Structure	Wavelength mμ Excitation, max	Fluorescence, max	Relative Fluorescent Sensitivity
Syrosingopine		300	360	4
Rescinnamine N-oxide		310	440	2–3
Syrosingopine N-oxide		290	350	6
Trimethoxybenzoic acid		280	360	2
Trimethoxycinnamic acid		300	400	<1
Indole		280	340	36
Norharmane		350 285	380 380	36 10
Harmaline hydrochloride		390	490	150

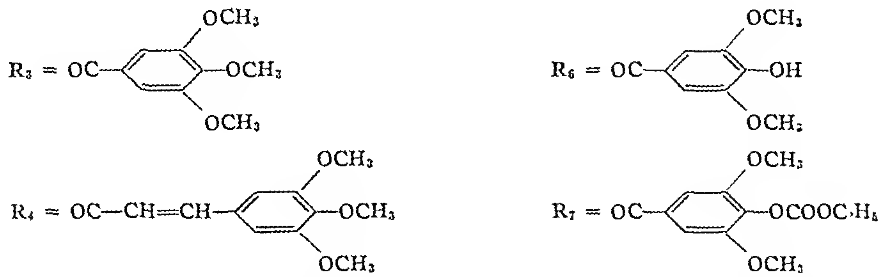
TABLE I—*Concluded*

Compound	Structure	Wavelength, mμ Excitation, max	Wavelength, mμ Fluorescence, max	Relative Fluorescent Sensitivity

Key:

R_1 = CH_3O R_5 = H

R_2 = $COOCH_3$



^a The authors wish to acknowledge the assistance of their associates, R A Lucas and P R Ulshafer, who supplied us with some of the alkaloids

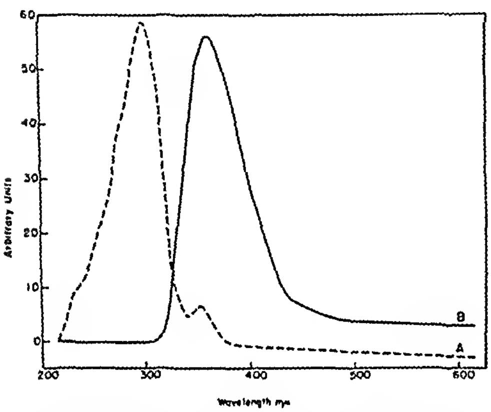


Fig 1—Excitation and fluorescent spectra of reserpine in methanol (3 μg /ml) A Excitation scan—fluorescence held at 360 mμ B Fluorescent scan—excitation held at 280 mμ

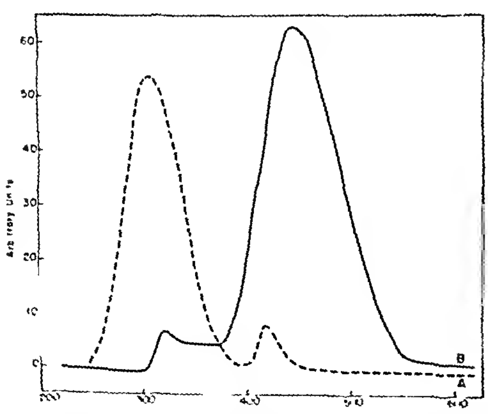


Fig 2—Excitation and fluorescent spectra of rescinnamine in methanol (3 μg , ml) A Excitation scan—fluorescence held at 440 mμ B Fluorescent scan—excitation held at 315 mμ

TABLE II—ANALYSES OF RESERPINE-RESCINNAMINE MIXTURES

Reserpine Added, %	Rescinnamine Added, %	Reserpine Found, %
99 2	0 8	98 5
99 2	0 8	99 2
95 2	4 8	95 2
95 2	4 8	94 2
80 0	20 0	80 0
80 0	20 0	81 5
40 0	60 0	40 9
25 0	75 0	25 8
16 7	83 3	17 2

tative difference can be utilized in the determination of reserpine in the presence of rescinnamine. However, it is not applicable when applied to preparations containing other rauwolfia alkaloids having identical fluorescent characteristics to that of reserpine unless used in conjunction with other techniques For example, methyl reserpate and reserpic acid may be removed by preliminary extraction with acid and base as outlined in the U. S P nitrite procedure (5) The presence of deserpidine, a possible contaminant of commercial reserpine obtained from *Rauwolfia canescens*, may be detected by chromatography (7) on paper and on columns or may be determined by difference between the spectrophotofluorometric technique and the U S P nitrite procedure (5), since deserpidine does not react with nitrous acid In spite of its limitations, the spectrophotofluorometry technique embodies certain advantages not inherent in other procedures and, in particular, may be applied to the determination of micro quantities of reserpine

Dehydroreserpine and 3-isoreserpine display a high visible fluorescence at 510 mμ, which is maximally excited at 390 mμ This shift in fluorescences of isoreserpine from the ultraviolet is not accompanied by a noticeable change in the absorption spectrum of the basic reserpine nucleus The fluorescence of dehydroreserpine is equivalent to twelvefold that of reserpine, and the fluorescent emission of iso-

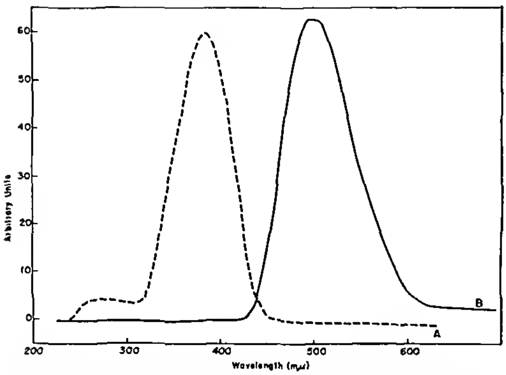


Fig. 3.—Excitation and fluorescent spectra of 3-dehydroreserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 510 mμ. B. Fluorescent scan—excitation held at 390 mμ.

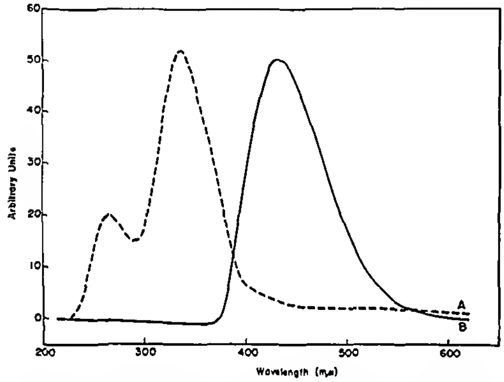


Fig. 4.—Excitation and fluorescent spectra of tetradehydroreserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 440 mμ. B. Fluorescent scan—excitation held at 340 mμ.

reserpine is increased by a factor of three over that of reserpine. Presumably, this shift in fluorescences is related to epimerization at C-3 and unsaturation in ring C. Although the significance of this phenomenon is not understood, it can be useful as a method for assaying reserpine, as the visible fluorescence of dehydroreserpine is increased 12 times that of the natural fluorescences of reserpine and offers the added advantage of the possibility of using filter type fluorometers. Details of a procedure based on the conversion of reserpine to dehydroreserpine to produce a fluoreseent peak at 510 mμ will be the basis of a subsequent publication.

An examination of tetradehydroreserpine, another oxidative degradation product of reserpine, likewise displays a sizeable increase of fluorescence over that of reserpine. In addition to the order of magnitude of fluorescence, the excitation and fluorescence emission are unlike those of either reserpine or dehydroreserpine. In this case the maximal fluorescent emission is found at 440 mμ when excited at 340 mμ.

CONCLUSIONS

1. A large number of rauwolfia alkaloids and related compounds have been subject to a spectrophotofluorometric study, and for each compound the wavelength of maximal excitation and fluorescence and relative fluorescent sensitivity are reported.

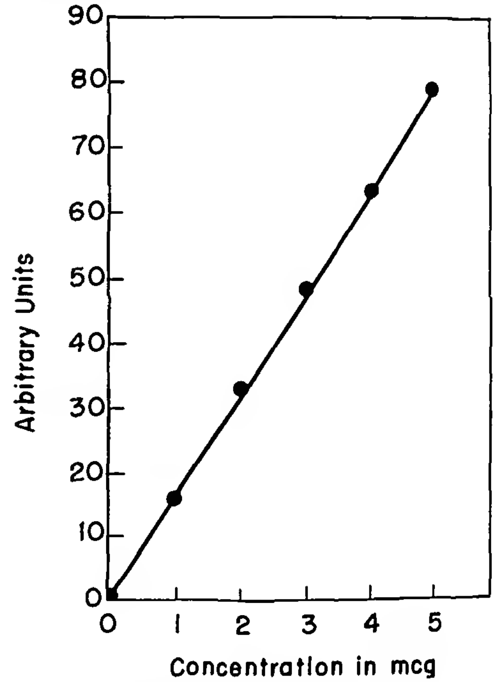


Fig. 5.—Relationship between concentration of reserpine and fluorescence.

2. The totality of the results of this survey allows several generalizations as to structural groups and fluorescence. The methoxy moiety and esterified substituent, trimethoxybenzoic acid, produces a decrease in the emitted light. The degree of fluorescence of indole alkaloids with a heterocyclic ring E is less than that of the reserpine nucleus. The introduction of oxygen at position 4 enhances the fluorescent emission. The substitution of trimethoxycinnamic acid as the esterified alcoholic function in place of trimethoxybenzoic acid is responsible for a shift as well as a weaker fluorescent response. Epimerization at C-3 and unsaturation in ring C shifts the fluorescence from the ultraviolet to the visible with an increase in the fluorescent intensity of several magnitudes.

3. A fluorometric procedure based on the conversion of reserpine to dehydroreserpine has been suggested. Details will be published later.

4. A practical spectrophotofluorometric procedure for the determination of reserpine in mixtures of reserpine and rescinnamine has been described that is based on the natural fluorescence of reserpine at 360 mμ when maximally excited at 280 mμ. Rescinnamine exhibits different excitation and fluorescent spectra and does not interfere.

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A Note on N-Ethoxy-N-ethyl-*m*-toluamide Insecticide*

By RANDOLPH T. MAJOR and HANS-JURGEN HESS†

Based on structural relationships it was postulated that N-ethoxy-N-ethyl-*m*-toluamide would prove to be an effective insect repellent as is N,N-diethyl-*m*-toluamide. It has been shown that N-ethoxy-N-ethyl-*m*-toluamide is an insecticide, particularly against mosquitoes.

RECENTLY, investigators in the U S Department of Agriculture have reported that N,N-diethyl-*m* toluamide is an active insect repellent (1–3)

Based on analogies between the pharmacological activity of substituted amines and substituted hydroxylamines (4) it seemed likely that N-ethoxy-N-ethyl-*m*-toluamide, m-$CH_3C_6H_4CON(C_2H_5)OC_2H_5$, would also be an insecticide or insect repellent

N-Ethoxy-N-ethyl-*m*-toluamide was prepared by the interaction of *m*-toluyl chloride (5) and N-ethoxy-ethylamine (6, 7)

EXPERIMENTAL

N-Ethoxy-N-ethyl-*m*-toluamide.—To 10 6 Gm (0 069 mole) of *m*-toluyl chloride in 10 cc of benzene was added, with external cooling, 13 Gm (0 146 mole) of N-ethoxy-ethylamine[1] in 10 cc of benzene After the mixture had stood, overnight, it was treated with an aqueous solution of potassium carbonate and extracted with ether The ether solution was dried over sodium sulfate and distilled, b p of the amide, 137–142° at 4 5 mm Redistilled, b p 139° at 4 5 mm , yield, 10 6 Gm (75%)

Anal —Calcd for $C_{12}H_{17}NO_2$: C, 69 51, H, 8 27, N, 6 76 Found: C, 69 60, H, 8 57, N, 7 05

Biological Activity.—Dr R F Phillips of the Merck Sharp and Dohme Research Laboratories has reported that the compound showed no antibacterial or antifungal activity against common plant pathogens However, he has written us that Dr Philip Granett of the Department of Entomology of Rutgers University put some mosquitoes, *Aedes aegypti*, in a five-inch long by two-inch diameter tube and tied a piece of cheesecloth which had been treated with a 5% solution of N-ethoxy-N-ethyl-*m*-toluamide in acetone on one end of the tube The mosquitoes were knocked down, i. e., paralyzed or killed rapidly Also, a moderate degree of repellency was noted against stable flies, *Stomoxys calcitrans*, but not against house flies, *Musca domestica* Dr Phillips has also written us that Dr. H. M. Peck of the Merck Institute for Therapeutic Research has carried out preliminary studies of the toxicity of N-ethoxy-N-ethyl-*m*-toluamide. These indicate that it is probably relatively nontoxic No indication of dermal injury to the skin of the rabbit was noted when the material was tested for twenty-four hours by the Draize patch test. Toxicity tests in Carsworth CF strain mice gave the following results:

i p LD_{50}—approx. 800 mg /Kg.
P O LD_{50}—approx. 2.0 Gm /Kg.

Toxic signs at such levels were loss of righting reflex, sedation, exophthalmia, ataxia, lacrimation, and slow respiration

SUMMARY

1 N-Ethoxy-N-ethyl-*m*-toluamide has been synthesized.

2 Preliminary toxicity tests indicate that N-ethoxy-N-ethyl-*m*-toluamide is relatively nontoxic systemically, and also to the skin.

3. N-Ethoxy-N-ethyl-*m*-toluamide shows considerable toxicity to mosquitoes, *Aedes aegypti*, and repells stable flies, *Stomoxys calcitrans*, but not house flies, *Musca domestica*

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* Received March 27 1959, from the Cobb Chemical Laboratory University of Virginia Charlottesville

The authors are indebted to Merck and Co , Inc , for a grant in support of this research and to the several investigators mentioned in this paper who have cooperated with them

† Merck and Co Inc , Post Doctoral Fellow in Chemistry, 1957–1958

[1] It should be noted that on one occasion a violent explosion occurred during the preparation of N ethoxy ethylammonium chloride by the method of R T Major and E E Fleck, *J Am Chem Soc* 50, 1479(1928) An aqueous solution of this salt was being concentrated *in vacuo* in an electric heating mantle Apparently the salt was overheated Aqueous solutions of N ethoxy ethylammonium chloride should be concentrated to dryness on a water bath or a steam bath, preferably *in vacuo*

Sensitivity to Vaginal Jellies: A Note on the Therapeutic Index of Spermicides*

By JOHN H. HOLZAEPFEL, JOHN S. WARNER, and JULIET A. HOWARD

In a recent publication (1) we described the development of a rabbit vaginal-irritation test that is recommended as a reliable procedure for the determination of irritating properties of vaginal jellies and creams. The present communication describes some of the results that we have obtained in using that procedure to evaluate the irritating properties and to determine the therapeutic indexes of experimental spermicides in jelly formulations.

Two main criteria of a satisfactory contraceptive jelly are effectiveness and safety. In an investigation to develop an improved contraceptive formulation, an extensive study (2) of the spermicidal effectiveness of chemical compounds was made by using a modification of the Brown and Gamble (3) screening procedure. A number of effective spermicides that had not been previously reported were discovered.

It was then necessary to determine the safety of these compounds in contraceptive formulations. In many cases the available toxicity data indicated that the materials were harmless. The rabbit vaginal-irritation test was used to determine another aspect of safety, namely, irritating properties. In contraceptive formulations as in most pharmaceutical products, the therapeutic index of an active ingredient must be considered as important as effectiveness alone. In our work, the therapeutic index was considered to be the ratio of the maximum nonirritating concentration in the rabbit vaginal-irritation test to the minimum concentration showing instantaneous immobilization of sperm in the modified Brown and Gamble test. Table I shows the results from the evaluation of a number of

Table I.—Data from Rabbit Vaginal-Irritation Test of Experimental Spermicides

Active Ingredient[a], %	Code No.[b]	Total Irritation Counts per Rabbit[c] Rabbit Number 1	2	3	4	5	6	Average
0.5 Lauramidine hydrochloride	12	20	1	15	10	18	15	13
0.5 Methylene dithiocyanate	19	44	47	44	34	29	44	40
8.0 *t*-Dodecylthionona(ethyleneoxy)ethanol	20	4	4	2	4	0	0	2
		0	1	0	0	3	0	1
8.0 *t*-Tridecoxypoly(ethyleneoxy)ethanol	21	0	0	0	0	0	0	0
4.0 *t*-Nonylphenoxypoly(ethyleneoxy)ethanol	29	0	0	0	5	1	0	1
		2	5	5	0	1	2	3
		7	3	0	3	0	7	3
4.0 Octylresorcinoxynona(ethyleneoxy)ethanol	32	5	2	2	0	5	1	3
		0	0	0	1	1	2	1
2.0 N,N,N′-Tris(hydroxyethylpolyoxyethylene)-N′-octadecyl-1,3-propylenediamine acetate	37	7	8	6	0	6	6	6
0.25 Bis(*n*-butylphenyl)iodonium chloride	51	23	14	1	7	6	6	10
Blank	.	0	0	1	4	0	0	1

[a] The active ingredient was formulated in a jelly base containing 2.0% boric acid, 10.0% glycerol, 0.02% butyl *p*-hydroxybenzoate, and 3.0% gum tragacanth. The pH was approximately 4.5.

[b] The code numbers that were used in a previous publication (2) are given here for comparison purposes.

[c] The irritation count is a measure of the various degrees of erythema, exudate, and edema observed on the days following each of three daily applications of jelly. Each type of irritation was graded as a 1, 2, or 3 degree level, depending upon its severity. Erythema was considered the mildest type of irritation. Each degree of erythema (designated as *E*) was given an irritation count of 1, i.e., $E_1 = 1$, $E_2 = 2$, $E_3 = 3$. E_1 was characterized by a slight redness of the mucosa, E_2 was characterized by redness accompanied by occasional distinct blood vessels, and E_3 was characterized by a deep redness accompanied by numerous engorged blood vessels. The presence of an exudate, composed of pus and/or sloughed tissue, was considered more severe than erythema but less severe than edema. Each degree of exudate (designated as *X*) was given an irritation count of 2, i.e., $X_1 = 2$, $X_2 = 4$, $X_3 = 6$. X_1 was characterized by a few small globules of pus, X_2 was characterized by large globules of pus, and X_3 was characterized by large amounts of pus and/or sloughed tissue exuding from the vulva. Edema was considered the most severe type of irritation. Each degree of edema (designated as *ED*) was given an irritation count of 3, i.e., $ED_1 = 3$, $ED_2 = 6$, $ED_3 = 9$. ED_1 was characterized by a slight swelling of the vulva, ED_2 was characterized by a swelling of the vulva to nearly twice its normal size, and ED_3 was characterized by a swelling of the vulva to several times its normal size. The various irritation counts for the three days were totaled for each rabbit. (For example, a rabbit showing E_1 on the first day, E_2, X_2, and ED_1 on the second day, and E_3, X_3, and ED_2 on the third day would be given irritation counts of 1, 9, and 15 for each of the three days, respectively, making a total of 25.) The highest total irritation count per rabbit possible is 54.

Table II.—Therapeutic Indexes of Experimental Spermicides

Code No.	Maximum Nonirritating Concentration,[a] %	Minimum Spermicidal Concentration,[b] %	Therapeutic Index[c]
12	0.25	0.5	0.5
19	0.1	0.1	1
20	8.0	0.5	16
21	8.0	0.5	16
29	4.0	0.5	8
32	4.0	0.5	8
37	1.0	0.5	2
51	0.1	0.1	1

[a] The maximum nonirritating concentration is an approximation of the maximum concentration that will result in an average irritation count of less than 5 in the rabbit vaginal-irritation test.

[b] The minimum spermicidal concentration is the minimum concentration that will result in an instantaneous immobilization of sperm in the modified Brown and Gamble test (2).

[c] The therapeutic index is considered here as the ratio of the maximum nonirritating concentration to the minimum spermicidal concentration.

* Received November 14, 1958, from the Department of Obstetrics and Gynecology, Ohio State University College of Medicine, and Battelle Memorial Institute, Columbus, Ohio.

This study was sponsored by the Research Department, Holland-Rantos Company, Inc., New York 13, N. Y.

effective spermicides (2) using the rabbit vaginal-irritation test (1) Table II shows the therapeutic indexes of those materials

Compound 51, for example, is five times as effective a spermicide as Compound 21; however, it has a much lower therapeutic index and thus would be much less satisfactory than Compound 21 as a contraceptive principle The importance of using the rabbit vaginal-irritation test in conjunction with a spermicide test for evaluating spermicides is, therefore, made apparent

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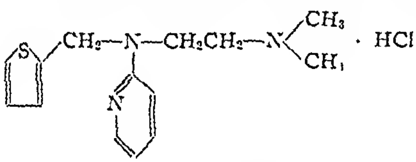
A Note on the Crystallography of Thenylpyramine Hydrochloride*

By HARRY A. ROSE and JOHN G. WILLIAMS

Thenylpyramine hydrochloride is a drug which is useful in the relief of allergic conditions Data are presented here which permit the identification of this compound by crystallographic methods

The chemical name of the compound is 2-[(2-dimethylaminoethyl)-2-thenylamino] pyridine and is represented by the following structural formula:



EXPERIMENTAL

Crystals suitable for crystallographic work may be obtained by recrystallization from isopropyl alcohol The crystals are monoclinic plates lying on the 100 face and closed by the 011 faces Occasionally small 210 faces may be seen

TABLE I —X-RAY DIFFRACTION DATA

Unit Cell Dimensions	a_0 = 27 35 A, b_0 = 10 38, c_0 = 10 96 A
Formula Weights per Cell	8
Formula Weight	297 85
Density	1 273 Gm /cc (flotation), 1 281 Gm /cc (X-ray)
Beta Angle	96°
Axial Ratio	$a:b:c$ = 2 635 : 1 : 1 056
Space Group	$C_{2}^{5}h - P2_1/n$

TABLE II —OPTICAL CRYSTALLOGRAPHIC DATA

Refractive Indexes (5893Å, 25°)	α = 1 592, β = 1 666, γ = 1 700, β' (in 100) = 1 642
Optic Axial Angle	(−) 2V = 65°40′ (calcd from α, β, and γ)
Orientation	$\beta \wedge c$ = 32° in acute β, $\gamma = b$

The X-ray powder diffraction data were obtained using copper radiation and nickel filter with a camera 114 6 mm in diameter. A wavelength value of 1.5405 *A* was used in the calculations. The indexing of the powder pattern was done on the basis of single crystal rotation patterns around both the *b* and *c* axis

On heating, thenylpyramine hydrochloride melts in the range 164 5–166 5°. The melt does not crystallize on cooling.

TABLE III —X-RAY POWDER DIFFRACTION DATA

d	I/I_1	hkl	d (calcd)
8 26	0 03	210	8 25
7 56	0 17	011	7 52
6 80	0 13	400	6 80
6 41	0 03	211	6 39
5 71	0 07	410	5 76
5 24	0 33	$\bar{2}02$	5 21
4 87	0 50	202, 220	4 89, 4 85
4 69	0 07	021	4 69
4 54	0 13	600	4 53
4 39	0 13	221	4 37
4 15	1 00	610	4 15
4 04	0 13	402	4 05
3 74	0 50b	412, 022, $\bar{6}02$	3 76, 3 76, 3 68
3 56	0 17	222	3 56
3 41	0 13b	620, $\bar{4}22$, 230	3 41, 3 40, 3 35
3 17	0 20	422	3 19
2 99	0 20	023	2 98
2 91	0 20	$\bar{8}12$	2 92
2 72	0 13	10, 00	2 72
2 62	0 07		
2 55	0 07		
2 50	0 07		
2 43	0 03		
2 40	0 07		
2 34	0 07		
2 29	0 03		
2 23	0 03		
2 13	0 07		
2 07	0 03		
2 04	0 03		
2 01	0 03		

* Received March 28 1959 from the Analytical Department Eli Lilly and Co Indianapolis 6, Ind

The United States Pharmacopoeial Convention, Inc.

ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the year ended December 31, 1958

STATEMENT OF INCOME AND EXPENSE

Income:			
Sales of pharmacopeias			
Collections		$39 098 75	
Less—Decrease in account receivable		3,960 25	$ 35,138 50
Sales of reference standards			
Collections		$41,964 18	
Add—Increase in accounts receivable and adjustment of prior years collections		1,346 12	43 310 30
Interest on investments less amortization of bond premiums of $22 65			6,839 85
Interest on savings deposits			6,489 61
Use of text by others			20 00
Miscellaneous			88 62
			$ 91 886 88
Expense:			
Revision		$95,722 15	
Less			
Increase in inventory	$ 905 43		
Opening of employees' pension trust account	300 00	1,205 43	$ 94,516 72
Administration			10,507 14
Publications		$ 1,023 39	
Add—Decrease in inventory		9 603 88	10,627 27
Headquarters		5,772 12	
Add—Depreciation expense			
Building	2,430 77		
Furnishings and equipment	4 146 68	6,577 45	12,349 57
			$128 000 70
Excess of Expense over Income			$ 30 113 82

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

Receipts:	
Sales of pharmacopeias	$39,098 75
Sales of reference standards	41,964 18
Interest on investments	6 862 50
Interest on savings deposits	6 355 21
Use of text by others	20 00
Miscellaneous	88 62
	$94,389 26

	Revision	Administration	Publications	Headquarters	Furnishings and Equipment	Total
Disbursements:						
Salaries	$57,416 77	$ 5,245 00		$1,675 00		$ 61 336 77
Meetings	5,567 87	115 21				5 683 08
Supplies	1,488 16	115 38				1 603 54
Postage and telegrams	1,642 69	50 33				1 693 02
Utilities				2 243 45		2 243 45
Repairs and maintenance				1 154 22		1,154 22
General	29,606 66*	4 981 22	$1 023 39			35 611 27
Insurance and taxes				699 45		699 45
Furnishings and equipment					$1 032 63	1 032 63
	$95 722 15	$10 507 14	$1 023 39	$5 772 12	$1 032 63	$111,057 40

* Retirement and Social Security	$11 502 78
Reference Standards (supplies only)	5,936 91
Research Assistants	10,847 29
Promotion	794 90
Miscellaneous	524 78
	$29 606 66

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVIII | *SEPTEMBER 1959* | NUMBER 9

Urinary Excretion Kinetics for Evaluation of Drug Absorption I*

Solution Rate Limited and Nonsolution Rate Limited Absorption of Aspirin and Benzyl Penicillin; Absorption Rate of Sulfaethylthiadiazole

By EINO NELSON and INGE SCHALDEMOSE

The use of urinary excretion data to evaluate drug absorption was discussed from the theoretical viewpoint and results of studies conducted to determine absorption of aspirin, benzyl penicillin, and sulfaethylthiadiazole were interpreted according to these considerations. It was shown that the absorption of aspirin was apparently rate limited by the time necessary for this drug to dissolve *in vivo* and that benzyl penicillin absorption appeared to be rate limited by the intrinsic solution rate properties of the salts used. The absorption rate of sulfaethylthiadiazole was calculated by means of excretion rate data and the results obtained shown to be in excellent agreement with calculation of the same quantity made from previously reported blood level data. The calculated absorption rate of this drug was about 1,200 mg./hr., fifteen to thirty minutes after the ingestion of 1-Gm. doses.

DRUG ABSORPTION STUDIES are usually conducted in the whole animal by measuring level of drug in blood or other fluids of distribution at various times after drug administration. This procedure does provide a direct basis for interpretations of the effect of variables introduced into experiments, but in some cases cannot be applied because small doses of drug result in less than assayable levels. In other cases, a vigorous and systematic sampling scheme may make it difficult to obtain test-subject participation. In still other cases, the information desired is of a comparative nature and direct determinations of blood levels are unnecessary. Urinary excretion data are frequently capable of supplying quantitative information on the absorption of drugs without the inconvenience of blood sampling even though these determinations are less direct. Since drug appearing in the urine may be considered concentrated therein as compared to the drug in circulation in the body fluids, then excretion data may be the only way in which the absorption of some drugs can be studied.

Information on urinary excretion has been used previously to evaluate the absorption of riboflavin (1, 2), sodium paraminosalicylate (3), chloramphenicol esters (4), aspirin, creatinine, and amphetamine on ion exchange resins (5), amphetamine in sustained release pellets (6), and tetracycline (7). This report reviews and discusses the theoretical bases for application of excretion data to evaluation of drug absorption, as well as the limitation of this application, and applies the concepts to show how *in vivo* solution rate rate-limits the absorption of aspirin and benzyl penicillin after oral ingestion, and to calculate the absorption rate of sulfaethylthiadiazole.

THEORY

Urinary excretion kinetics are one part of the broader field of the kinetics of drug absorption, distribution, metabolism, and excretion and a substantial amount of information is available in the litera-

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ture in this general field (8-26) The usefulness of urinary excretion measurements lies in the direct proportionality which exists between excretion rate and amount or concentration of drug in the blood stream [1] When drug in blood is in equilibrium with the other fluids of distribution, then excretion rate is proportional to the concentration or amount in all fluids of distribution The proportionality may be expressed as (6)

$$\frac{dAe}{dt} = KfA_b \qquad \text{(Eq 1)}$$

where dAe/dt is excretion rate in amount per unit time, K is the rate constant for removal from the body in reciprocal time, f is the fraction of administered drug excreted unchanged in urine, and A_b is the amount of drug in the blood or in the body, depending on whether or not blood and other fluids of distribution are at equilibrium Equation 1 may be written as well in terms of concentration in the blood since A_b is equal to the product of blood concentration and distribution volume, C_b and V_d, respectively At equilibrium between blood and other fluids of distribution, V_d represents total volume of distribution Equation 1 written in this form was the basis for determination of volume of distribution by simultaneous measurement of excretion rate and blood level (15) In any application of Eq 1, it is implicitly understood that no changes are made in test conditions that would cause a change in renal clearance of the compound under study

Equation 1 is equally valid when applied to the excretion of a metabolite of a drug For example, if drug A is administered and it is transformed partially or entirely to a metabolite B, then Eq 1 may be used to plot the amount *vs* time course of the metabolite in the blood stream This would, of course, require information on the value of the constants K and f and further, assurance that the site of the metabolic conversion is such that the metabolite enters the general circulation If the rate of the conversion A to B is known, then, conceivably, excretion data on the metabolite could be used to elucidate the amount *vs* time course of its precursor A in circulation This would probably not be practical in most cases due to difficulties of measurement of the necessary quantities

The application of Eq 1 to evaluation of drug absorption is particularly direct when the information desired is of a comparative value For example, if the absorption of a given drug in several salt forms is desired to be compared, then the ratio of excretion rates, R,

$$R = \frac{(A_b)_1}{(A_b)} \qquad \text{(Eq 2)}$$

is the ratio of amounts of drug from forms 1 and 2 in the blood Since the same drug is under consideration, the constants K and f are equal and cancel when the ratio is taken (6)

Application of Eq 1 in the manner just described is also possible when the excretion of a metabolite is followed The quantity R, of course, then refers to the ratio of the amounts of metabolite in circulation

When the constants K and f are known in Eq 1, then the actual amount in circulation may be calculated With information as to the distribution volume of the drug, this information is reducible to concentration in the fluids of distribution The limitation in this application lies in finding the excretion rate over relatively short periods of time This information is probably most easily obtained by graphical determination of the slope of a plot of cumulative amount of drug excreted with time The accuracy of the values so obtained depend both on the carefulness with which the graphical constructions are performed as well as the accuracy and precision of the assay conducted to determine urine concentration

Equation 1 has been used as the basis for development of an expression to calculate absorption rate in terms of amount per unit time (27) The expression had the following form

$$\text{Absorption Rate} = \frac{1}{f}\left(\frac{dA_e}{dt} + \frac{d^2A_e}{Kdt^2}\right) \qquad \text{(Eq 3)}$$

The second derivative term may be found graphically from a plot of excretion rate *vs* time The determination of K and f and the limitations in application of Eq 3 have been discussed (27)

Additional theoretical considerations on interpretation of excretion data have been discussed (6, 7, 27)

EXPERIMENTAL

Conduction of Tests.—All absorption tests were conducted using adult humans in apparent good health All drugs were ingested orally on fasting stomachs in the morning No food was taken by subjects until at least one and one-half hours after ingestion of drug Urine blanks were obtained immediately before drug ingestion Five to seven subjects were used in tests depending on the drug under study, and their participation in a given test as well as their ages and weights are indicated on the tabulated data which follow later in this report These data also indicate the urine sample collection times with the several drugs studied

Preparation of Dosage Forms.—*Aspirin*—Tablets of aspirin containing 325 ± 5 mg drug were prepared from a standard precompressed granulation of aspirin containing 10% starch [2] The test set of tablets was compressed at 5,000 p s i These tablets were $^3/_8$ inch in diameter and made with standard cup punches in a Carver press modified for tablet compression Two tablets of the set constituted a dose in the absorption studies In addition, 650 ± 5 mg aspirin of U S P XV quality was used in the tests after dissolving this amount in about 250 ml water After this dose in solution was administered, the container was rinsed at least once and the rinsings also ingested

Sulfaethylthiadiazole—This drug was taken from a sample of commercial purity [3] and 1,000 ± 25 mg constituted a dose The drug was ingested after suspension in about 250 ml of water

Benzyl Penicillin Salts—The potassium and procaine salts of this drug were of a grade suitable for parenteral use A 62 5 ± 2 5 mg dose of the po

[1] Exceptions exist in cases where there is a threshold level such as with glucose and in cases where the body level is at such a high value as to saturate function The latter case would ordinarily not occur with the usual range of drug doses

[2] Merck and Co Inc Rahway N J
[3] Kindly supplied by Dr Stanton Hardy, Lederle Laboratories Division American Cyanamid Co, Pearl River, N Y

tassium salt and 100 ± 5 mg.-dose of the procaine salt were used in the absorption study. These were administered as thin, flat-faced pellets 0.95 cm. in diameter, contained in 000 hard gelatin capsules with about 50 mg. sodium bicarbonate in each end. Pellets were chosen as to weight so that one pellet constituted a dose. The pellets were prepared by a compression technique previously described (7). No binders, disintegrating agents, diluents, or lubricants were incorporated before compression. The pellets were hard and homogenous and resisted disintegration in water for several hours (the procaine salt—the potassium salt dissolved rapidly from its exposed faces). The administration of benzyl penicillin in the form described insured that tests were conducted under conditions of essentially equal and constant surface.

Analytical Methods.—Aspirin was determined as salicylic and salicyluric acids colorimetrically using a ferric nitrate reagent (28). Benzyl penicillin was determined by cylinder-plate assay, using *Staph. aureus* 209 P as the test organism (29).[4] Sulfaethylthiadazole was determined colorimetrically by the Bratton and Marshall method. All absorbance determinations were made with a Bausch and Lomb Spectronic 20 colorimeter. Blank urine samples were assayed and appropriate corrections made when necessary.

RESULTS AND DISCUSSIONS

Aspirin Absorption in Solution and Tablets.—Excretion data from aspirin ingestion in tablets and in solution are shown in Tables Ia and Ib. The nature of the cumulative amounts excreted *vs.* time curves is shown on Fig. 1. All data were in terms of apparent amount of salicylate excreted since the assay method determines both unchanged salicylic and salicyluric acids (30). Since there was no reason to believe that the excretion ratio of these two substances changed in a given subject from test to test the comparison of amounts of apparent salicylate excreted in the two cases was a valid basis for comparison of aspirin absorption from the two dosage forms. Aspirin anion is very rapidly hydrolyzed to salicylate anion in the plasma (31), so this occurrence did not affect the comparison of absorption from the two dosage forms.

It has been calculated that the time needed to effect solution of aspirin at absorption sites in the gastrointestinal tract should rate limit absorption of this drug (32). The theoretical calculation was based on the area of absorbing surface expected to be available in the gastrointestinal tract, diffusion coefficient of aspirin, and other pertinent quantities involved (32). In the present work, the maximum mean excretion rates of salicylate were greater from aspirin in solution (Table Ic) than they were from aspirin in rapidly disintegrating tablets at comparable times. As a consequence of Eq. 1, the salicylate level in blood was greater from the solution dosage also. The present work, then supports the theoretical calculation. Data in the literature on salicylate levels in blood from dosage in solution and in tablets give additional support to the theoretical calculation (33). With regard to the data of Table I, the t-test showed that the difference in amounts excreted in the two dosage forms was significant at four hours at the 99% confidence level.

The rate-limiting nature of the *in vivo* solution step should be considered in any assessment of drug absorption when the change in experiment is concerned with change in dosage form or salt form of a particular drug (34, 35). Recognition of the importance of this factor should in some instances eliminate the necessity for experiment, or at least allow its conduction with an awareness of the possible effect of an important variable

Benzyl Penicillin Absorption from the Potassium and Procaine Salts.—Excretion data from tests with these salts are listed in Tables IIa and IIb. The mean cumulative excretion curves are shown on Fig. 2. The differences shown were expected since the method of dosage form preparation insured that the rate limiting factor in absorption would be the intrinsic solution rate properties of each salt. These results were completely in accord with results obtained from blood level studies (42) but interpretation of the present results were less ambiguous, since

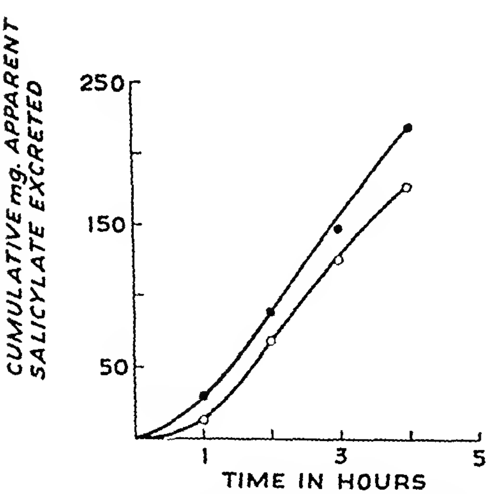

Fig. 1.—Mean cumulative amounts of apparent salicylate excreted to various times after ingestion of 0.65 Gm. aspirin in solution and in rapidly disintegrating tablets. Solid points, drug in solution; open points, drug in tablets

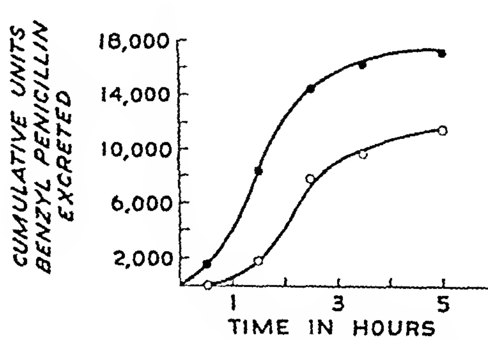

Fig 2—Mean cumulative amounts of benzyl penicillin excreted to various times after ingestion of 100,000-unit doses of the potassium and procaine salts in the form of limited and constant surface area pellets. Solid points, potassium salt; open points, procaine salt.

[4] The assistance of Mrs. Yoko Yuzuriha is gratefully acknowledged

TABLE Ia.—CUMULATIVE MG. SALICYLATE AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS FOLLOWING INGESTION OF 0.65 GM. ASPIRIN IN SOLUTION[a]

Subject[b]	Excreted, mg.				Urine, ml.			
	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
O (28-68)	25.5	85.0	139.0	189.0	40	360	550	590
M (28-68)	27.5	81.0	149.0	213.0	300	650	960	1,060
N (21-77)	29.6	88.8	147.0	216.0	150	480	770	900
X (22 58)	20.6	65.0	111.9	223.5	310	490	650	830
P (26-75)	45.6	123.4	189.6	251.2	120	220	270	310
Mean	29.8	88.6	147.3	218.4	184	440	640	738

[a] Apparent salicylate—see text. [b] Bracketed quantities are subject's age in years followed by his weight in kilograms.

TABLE Ib.—CUMULATIVE MG. SALICYLATE AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS FOLLOWING INGESTION OF 0.65 GM. ASPIRIN IN TABLETS[a,b]

Subject	Excreted, mg				Urine, ml.			
	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
O	21.8	69.5	128.5	182.0	80	440	510	560
M	0.6	60.1	118.7	167.1	40	190	330	390
N	18.4	59.2	107.6	166.9	220	340	380	660
X	21.0	82.5	144.5	196.5	180	380	540	700
P	19.2	72.5	130.0	176.5	70	110	150	190
Mean	12.4	68.8	125.9	177.8	118	292	582	500

[a] Apparent salicylate—see text. [b] Mean disintegration of eight by U. S. P. XV method equal to four seconds.

TABLE Ic.—EXCRETION RATES OF APPARENT SALICYLATE AFTER INGESTION OF 0.65 GM. ASPIRIN IN SOLUTION AND IN RAPIDLY DISINTEGRATING TABLETS[a,b]

Time, hr.	Excretion Rate, mg./hr. Solution	Tablets
0.5	30	10
1.0	45	35
1.5	58	42
2.0	75	65
2.5	70	57
3.0	62	50
3.5	57	45

[a] Computed from data on Fig. 1 by graphical determination of slopes at indicated times.
[b] Values shown determined over half-hour intervals having indicated times at their midpoint.

it could be argued that the effects observed in the cited work might be due to differences in drug release properties of the dosage form.

It has been shown previously that the salts of the same acid may dissolve at widely different rates (34–36). The rapid *in vivo* solution rate of the potassium salt of benzyl penicillin was due to the properties of the diffusion layer that surrounds this material (or other potassium or sodium salts of weak acids) as it dissolved. The Noyes–Whitney solution rate law states that

$$\frac{da}{dt} = KS(C_s - C) \qquad \text{(Eq. 4)}$$

where da/dt is the solution rate, K a constant, S the surface area, C_s the concentration of a saturated solution of the dissolving species, and C the concentration in the dissolution medium at any time. If *in vivo* solution took place in the stomach when the pellets of potassium benzyl penicillin were ingested, then Eq. 4 could be written to describe dissolution therein by substituting for C_s as

$$\frac{da}{dt} = KS(C_o(1 + K_a/[\mathrm{H}^+]_d) - C) \qquad \text{(Eq. 5)}$$

where C_o is the solubility of the undissociated acid (37), K_a the acid's dissociation constant, and $[\mathrm{H}^+]_d$ the hydrogen ion concentration of the diffusion layer. This latter quantity would be the hydrogen ion concentration of a saturated solution of potassium benzyl penicillin in gastric fluid which would be essentially that of a saturated solution of this salt in water, since preparation of the saturated solution would necessitate adding the salt without limit to either fluid and in the case of gastric fluid, its acid content would be neutralized. In acidic gastric fluid, C would be essentially equal to C_o and Eq. 5 reduces to

$$\frac{da}{dt} = KSC_o\, K_a/[\mathrm{H}^+]_d \qquad \text{(Eq. 6)}$$

Equation 6 should also hold in more alkaline regions of the gastrointestinal tract since absorption constantly decreases drug titer in the fluids there and this process should allow equation of C to C_o.

Equation 6 should also be applicable to description of the *in vivo* dissolution of the procaine salt of benzyl penicillin in gastric fluid. However, in the case of this salt the diffusion layer pH would be much nearer that of the medium, since it would be represented by a saturated solution of procaine benzyl penicillin in gastric fluid. At equilibrium this would be the hydrogen ion concentration of a saturated solution of procaine hydrochloride, benzyl penicillin free acid, and some anion. It seems reasonable to expect that the hydrogen ion concentration in the diffusion layer in the two cases would differ by at least one hundred-fold, hence the *in vivo* solution rates should differ by the same factor. This difference must be the explanation for the difference in absorption, as reflected in excretion rates observed soon after ingestion of drugs (Table IIc). The degree of difference in excretion rates would no doubt be much greater if benzyl penicillin were stable in acid medium. The pellets of its potassium salt could be expected to have dissolved completely in the stomach and this was probably not the case with the procaine salt because of its slower solution rate. Substantial amounts of penicillin from the potassium salt would be degraded since the half-life of benzyl penicillin at pH 2 and 37° is of the order of ten minutes (38).

TABLE IIa.—CUMULATIVE UNITS BENZYL PENICILLIN AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS AFTER INGESTION OF 100,000 UNITS OF POTASSIUM SALT[a, b]

Subject[c]	Units Excreted 0.5	1.5	2.5	3.5	5.0	Urine, ml. 0.5	1.5	2.5	3.5	5.0
M (28 63)	0	8,600	20,800	24,300	26,300	20	160	450	640	910
E (39 77)	3,100	10,100	13,200	13,700	14,000	60	110	180	250	420
S (33 57)	2,700	6,700	9,000	9,700	10,200	40	70	170	300	420
P (26 75)	900	8,200	10,200	10,800	11,600	40	120	240	310	340
I (33 61)	700	8,700	18,900	22,400	23,600	30	80	120	150	230
Mean	1,480	8,460	14,420	16,180	17,140	38	108	232	330	464

[a] Dose contained in a single pellet 0.95 cm. in diameter—see text.
[b] Shown to the nearest 100 units.
[c] Bracketed quantities are subject's age in years followed by his weight in kilograms.

TABLE IIb.—CUMULATIVE UNITS BENZYL PENICILLIN AND ML. URINE EXCRETED TO VARIOUS TIMES IN HOURS AFTER INGESTION OF 100,000 UNITS OF PROCAINE SALT[a, b]

Subject	Units Excreted 0.5	1.5	2.5	3.5	5.0	Urine, ml. 0.5	1.5	2.5	3.5	5.0
M	0	2,400	7,900	10,100	14,800	20	80	150	250	430
E	0	1,300	2,900	3,200	3,400	30	340	550	620	690
S	0	1,600	4,400	8,200	10,900	10	60	230	520	810
P	0	1,600	2,700	3,400		20	100	180	210	
I	0	2,900	21,200	23,200	24,500	20	110	270	310	410
Mean	0	1,960	7,820	9,600	13,400	20	138	276	382	585

[a] Dose contained in a single pellet 0.95 cm. in diameter—see text. [b] Shown to the nearest 100 units.

TABLE IIc.—EXCRETION RATES OF BENZYL PENICILLIN AFTER INGESTION OF 100,000 UNITS OF THE POTASSIUM AND PROCAINE SALTS IN THE FORM OF LIMITED AND CONSTANT SURFACE AREA PELLETS[a]

Time, hr.	Excretion Rate, Units/hr. Potassium salt	Procaine Salt
0.5	4,000	400
1.0	6,800	1,600
1.5	8,800	3,600
2.0	6,000	6,400
2.5	3,600	4,600
4.0	800	1,200

[a] Mean rates computed from curves on Fig. 2 by graphical determination of slopes at indicated times.

Differences in absorption rate have been noted many times when blood levels from administration of slightly soluble weak acids as the acid or slightly soluble salt have been compared to the blood levels obtained from the administration of the same acid as the potassium or sodium salt. The results obtained in the present work with benzyl penicillin are in accord with other studies using the same compounds (42). Higher blood levels were obtained from administration of the sodium and potassium salts of para-aminosalicylic acid as compared to the free acid (43), from the sodium salts of sulfapyridine and sulfathiazole as compared to their respective free acids (44), from the sodium salt of novobiocin as compared to the free acid (45), from the potassium salt of phenoxymethyl penicillin as compared to the free acid (46), and it has also been observed that hippuric acid was excreted at a more rapid rate when sodium benzoate was ingested as compared to the excretion rate when benzoic acid was ingested (47). These observations along with the present work validate the *in vitro* solution rate explanation. Other considerations on this topic were discussed (35).

The comparative absorption kinetics in the case of the salts of benzyl penicillin are easily interpreted with the aid of Eq. 2 from data in Table IIc. The average blood level ratios, potassium to procaine salt, at various times are listed below:

Time, hr.	R
0.5	10.0
1.0	4.2
1.5	2.4
2.0	0.93
2.5	0.78
4.0	0.67

The literature (39) on penicillin distribution and excretion in a normal human contains information that allows calculation of the constants K and f in Eq. 1. The half-life of benzyl penicillin in circulation was about 0.46 hours which allows assignment of 1.5 hours^{-1} as the value of the constant K. Further, it appeared that about 80% of injected penicillin was excreted as unchanged material, allowing the assignment of 0.8 for the value of f. These values allow writing Eq. 1 as $da_e/dt = 1.2 A_b$. Since the maximum excretion rate from the potassium salt was 8,800 units/hour, the maximum amount of penicillin in circulation was about 7,300 units. The penicillin data (39) may be interpreted in terms of a relationship derived previously (15) to determine a penicillin volume of distribution of about 19 L. In the present case, then, the maximum concentration from the potassium salt was about 0.38 units/ml. This value was about one-half of the reported maximum blood levels after 200,000-unit oral doses of potassium benzyl penicillin in capsules in humans (40). The bases for these calculations were from limited data and were only included to illustrate full application of Eq. 1.

Absorption Rate of Sulfaethylthiadiazole.—The tabulated data of Table III show the amounts of this drug excreted to various times. These data were used to calculate its absorption rate by Eq. 3 since data were available in the literature to calculate absorption rate for this drug from blood level measurements (41). An agreement between calculated values would tend to show the essential soundness of the excretion rate approach to study drug absorption.

The means of the cumulative amounts excreted data shown in Table III are plotted against time on

TABLE III —CUMULATIVE MG SULFAETHYLTHIADIAZOLE AND ML URINE EXCRETED TO VARIOUS TIMES IN HOURS AFTER INGESTION OF 1 0 GM DRUG IN SUSPENSION[a]

Subject[b]	Drug, mg						Urine, ml					
	0 5	1 5	2 5	3 5	6 0	8 0	0 5	1 5	2 5	3 5	6 0	8 0
O (28-68)	5 4	58 0	141 0	246 3	453 3	565	60	470	530	580	730	810
M (28-63)	5 8	69 5	144 5	269 0	428 0		80	570	730	840	900	
P (26-75)	0	88 4	181 9	275 3	353 8	405 5	20	120	300	400	510	560
E (39-77)	14 8	101 8	229 6	307 8	465 0	550 5	190	790	970	1,060	1,240	1,390
S (33-57)	14 5	123 2	232 4	308 4	414 0	495 2	60	430	690	790	900	1,040
N (21-77)	2 0	50 1	106 8	166 3	250 3	305 7	80	500	570	650	770	910
I (33-61)	42 5	196 9	277 2	363 1	442 3	589 1	40	170	270	340	410	570
Mean	12 1	98 3	187 6	276 6	401 0	485 2	76	436	580	666	780	880

[a] Values shown are for free drug Only negligible amounts of acetylated drug were excreted to the longest time shown
[b] Bracketed quantities are subject's age in years followed by his weight in kilograms

Fig 3 Values for the excretion rate term of Eq 3 were obtained from a similar plot only with an expanded scale for excretion between zero and three and one-half hours These values were mean rates over twelve-minute intervals having the times listed in Table IV as their midpoints The rates obtained by this graphical determination are shown in the second column of Table IV The second derivative terms for Eq 3 were obtained by plotting these rates *vs* time and were taken over the same time interval The values obtained are listed in the third column of Table IV The value of the constant K in Eq 3 was obtained from the mean half-lives for elimination of sulfaethylthiadiazole by the subjects as determined by plotting their graphically determined individual excretion rates, at four and one-half, five and one-half, six and one-half, and seven and one half hours after drug ingestion, as a function of time on semilogarithmic paper[5] The half-lives so obtained are listed in Table V The value of the constant K obtained by use of the mean half-life was 0 091 hours^{-1}. The only other quantity needed to calculate absorption rate was the value of f which was taken at 0 92 since this value appears in the literature as the fraction of this drug in circulation that is excreted as unchanged material (41) The calculated absorption rate in mg /hour at various times is shown in column 4 of Table IV

TABLE IV —ABSORPTION RATE OF SULFAETHYLTHIADIAZOLE FROM URINARY EXCRETION DATA AND BLOOD LEVEL DATA[a]

Time, hr	Excretion rate, mg /hr	Derivative of Excretion Rate, mg /hr ^{2}	Absorption Rate from Excretion Data[b] mg /hr	Absorption Rate from Blood Data[b] mg /hr
0 25	20	107	1,200	500
0 50	50	105	1,210	1,230
0 75	72	83	1,000	1,170
1 00	87	55	700	830
1 25	100	35	490	250
1 50	112	0	120	90

[a] Derived data needed to calculate absorption rate from urinary excretion data are also shown The calculation in the fourth column was made by Eq 3
[b] Nearest 10 mg /hr

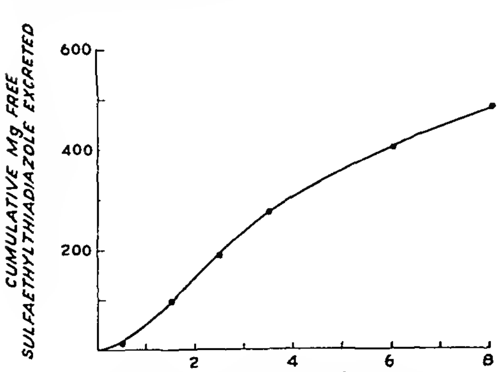

Fig 3 —Mean cumulative excretion curve for sulfaethylthiadiazole after ingestion of 1 0-Gm doses by seven subjects Drug taken in suspension

TABLE V —HALF-LIVES FOR ELIMINATION OF SULFAETHYLTHIADIAZOLE BY TEST SUBJECTS[a]

Subject	Half Life, Hours
O	9 7
P	3 1
E	9 1
S	7 8
N	5 4
I	10 8
Mean	7 6

[a] See text for method of determination

A method of calculating absorption rate based on blood level measurement at short-time intervals following drug ingestion (17) was applied to data in the literature obtained following the same dose of sulfaethylthiadiazole (41). The blood level data on eight normal human subjects were averaged and the absorption rate calculated at the same times with rates determined over the same time intervals as in the calculation based on Eq 3 The values so calculated are shown in column 5 of Table IV[6] The agreement in calculated rates was surprisingly good A different particle size distribution may have existed in the two sets of dosage forms, but the sulfaethylthiadiazole was from the same manufacturer The conditions of administration differed to the extent that, while subjects in both groups took the drug on fasting stomachs, the drug was ingested in suspensions for urinary excretion measurements and in capsules for blood level measurements. The lower value in absorption rate calculated at fifteen minutes (Table IV) from capsule dosage may reason-

[5] Excretion rates at earlier times were not used in this plot to avoid introduction of error due to continued absorption of small amounts of drug and/or nonequilibrium conditions between blood and other distribution fluids

[6] The data used were from Table I of (41) for 1 0 Gm doses Other constants used in this calculation which were in accord with information in the cited paper were Vb = 15 000 ml (based on mean body weight of subjects) and kb = 0 092 hours^{-1} The quantities, Vb and kb, are in the terminology of (41)

ably be attributed to a five to ten-minute delay in the capsules' contents reaching the same state of dispersion *in vivo* obtained when the drug was taken in suspension

The area under an absorption rate *vs* time curve plotted from data obtained by Eq 3 gave an apparent amount of drug absorbed equal to 1 17 Gm

SUMMARY

The theoretical bases for application of urinary excretion data to evaluation of drug absorption were reviewed and the concepts applied to study (*a*) the absorption of 0.65-Gm doses of aspirin in solution and in rapidly disintegrating tablets, (*b*) the absorption of 100,000 units of benzyl penicillin from the potassium and procaine salts given in thin pellets of limited surface area wherein *in vivo* solution rate, rate-limited absorption, and (*c*) the absorption rate of sulfaethylthiadiazole following 1 0-Gm doses All studies were conducted in normal humans after oral ingestion of drugs The absorption of aspirin appeared to be rate limited by the time necessary for this drug to dissolve after ingestion and this finding was in accord with theoretical calculations previously made which indicated that this process should be rate limiting in absorption The absorption of benzyl penicillin was found to be rate limited by the intrinsic solution rate properties of the potassium and procaine salts used. The *in vivo* solution rates of these two salts were considered on a physical chemical basis and an expression derived to relate rate to diffusion layer pH From excretion studies with sulfaethylthiadiazole it was calculated that this drug was absorbed fifteen to thirty minutes after ingestion, at a rate of about 1,200 mg / hour This value was in excellent agreement with absorption rate calculated from blood level data appearing in the literature

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The Effect of Gibberellic Acid on the Second-Year Growth and Alkaloid Formation in *Atropa belladonna* Linné*

By LEO A. SCIUCHETTI

Atropa belladonna treated in 1957 with gibberellic acid in concentrations of 100 and 1,000 p. p. m. was allowed to overwinter. The second-year crop of the treated plants generally did not demonstrate the "gibberellin effects" noted in the first-year growth following treatment. In some instances reverse effects were noted. The plants previously treated with 100 p. p. m. of gibberellic acid generally closely resembled the controls in their growth and alkaloid patterns. However, belladonna treated the previous year with 1,000 p. p. m. of gibberellic acid was profoundly affected. This treatment induced about a 50 per cent decrease in total dry weight and approximately a 15 per cent decrease in height. The concentration of alkaloids in the leaves and roots was not markedly altered, whereas that in the stems was increased about 44 per cent. The total alkaloid content of this group was about 56 per cent that of the controls.

IN A PREVIOUS PUBLICATION (1) it was demonstrated that the growth and alkaloid content of *Atropa belladonna* were adversely affected by treatment with gibberellic acid (G. A.). This study was a continuation of that research in that the remaining plants (treated and untreated) were allowed to overwinter. The objective of this research was to observe whether the characteristic "gibberellin effects" would again be manifested in the second-year growth.

EXPERIMENTAL

Procedure.—Fifty-four belladonna plants remaining from the previous study (1) were allowed to overwinter in the drug garden. The plants were divided into three groups of 18 plants each. The first group constituted the control or untreated plants; the second were those subjected in 1957 to a three-spray treatment with 100 p. p. m. of G. A.; and the third were those similarly treated with 1,000 p. p. m. of G. A. The first-year growth continued until mid-December, 1957, when a killing frost occurred. Several weeks of freezing temperatures were followed by about a month of warm moist weather. Due to the mild winter new shoots appeared during the second week in February, 1958. Height measurements were started about two months later. On June 3, 1958, ten plants were picked at random from each group and the shoots were harvested. At this time the plants were at the flowering stage. This constituted the first crop.

Following the first harvest the plants were fertilized by the application of a complete organic fertilizer[1] to the plot containing the plants. Height measurements for the second crop were commenced on June 23. The plants were harvested on September 9. At this time the plants were in the flowering and fruiting stage, i. e., many of the plants had green berries.

The first crop, consisting of the shoots, was harvested and divided into two portions—leaves (with flowering tops) and stems. Fresh weights were immediately taken of each portion and the fresh material was promptly transferred to a hot-air circulating drier. The plant parts were dried at a temperature of 48.5° for forty hours. Upon removal from the drier the plant parts were placed in a desiccator, allowed to attain room temperature, and then weighed. The portions were stored in air-tight metal containers until subsequent pulverization into a No. 40 powder in a Wiley mill. The powdered material was then stored in air-tight colored-glass containers until subsequent analyses for alkaloids were performed. For the second crop, an additional (third) portion which consisted of the roots was harvested. Otherwise, the harvesting procedure was identical with that for the first crop.

Growth Observations.—New shoots mainly in the control group were first observed during the second week of February, 1958. All of the control plants survived the winter. One plant from the group treated with 100 p. p. m. of G. A. and two from the group treated with 1,000 p. p. m. of G. A. did not overwinter. In general, the untreated plants and those previously treated with 100 p. p. m. of G. A. closely resembled each other in growth characteristics during the growing season. However, those previously treated with 1,000 p. p. m. of G. A. did not commence to form shoots from the first-year root stocks until about two weeks after the controls. Growth in this group was slower and the onset of flowering was later. Also, the type of growth was somewhat different in that the plants were spindly and vine-like and the leaves were narrower and thinner.

On April 16, 1958, height measurements were taken of all the plants. These measurements were taken weekly thereafter at approximately the same time of the day. For each crop, the control plants and those treated with 100 p. p. m. of G. A. grew to approximately the same height (Fig. 1). However, the first crop of the group treated with 1,000 p. p. m. of G. A. attained a height of about 61% of the controls at the start of measurements and about 83% of the controls when harvested. The second crop of this group attained about the same height as the controls at the start of measurements on June 23, but

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[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis—5% of total nitrogen, 3% available phosphate, 2% available potash.)

were about 85% as tall as the controls when harvested (Fig 1)

Correlation With Fresh and Dry Weight Data.—The treated plants displayed decreased dry weights in their second-year growth (Table I) The plants previously treated with 100 p p m of G A displayed slight decreases of about 8 to 10% in the dry weights of their organs. This consistency was noted for both crops Significant decreases, however, were observed in the plants previously treated with 1,000 p p m of G A Their total dry weight was about one-half that of the controls The decreases were considerably greater for the plants of the second crop Also, the roots displayed the largest decrease among the organs of this group (Table I)

Analysis for Alkaloids.—The dried plant parts, using pooled samples, were assayed for total alkaloids (expressed as hyoscyamine) according to the Witt-Youngken method (2), substituting chloroform for benzene as the immiscible solvent The concentration of alkaloids in the various plant organs of the group treated with 100 p p m of G A differed slightly from that of the controls (Table II) This was also true for the leaves and tops and the roots of those treated with 1,000 p p m of G A However, significant increases were found in the stems of this group. Compared with controls, the increases were about 28% and 54% for the first and second crops, respectively. The increase for the total crop of stems was about 44%

It was further desired to make comparisons based on the total alkaloid content per plant and per plant organ The total alkaloid content was obtained by multiplying the dry weight of the plant organ by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in mg (Table II) All of the plants treated with 100 p p m of G A contained less total alkaloids than the controls The decreased alkaloid content of this group was not considered significant Significant decreases, however, were found in the plants treated with the higher concentration of gibberellic acid In general, the total alkaloid content of this group was about one-half that of the controls The roots displayed the greatest decrease among the plant organs, *viz*, 57% less than the controls

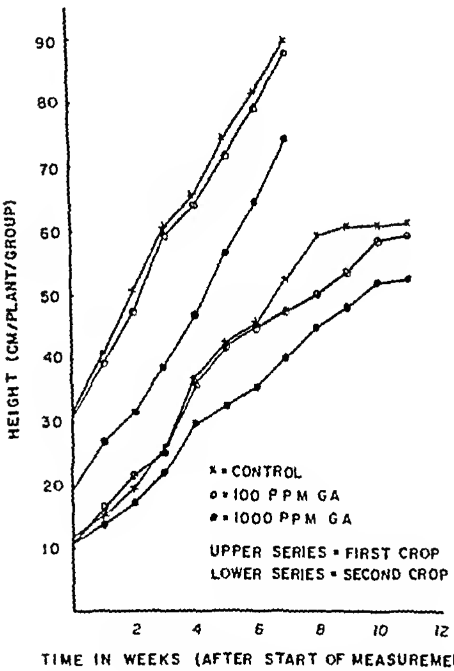

Fig 1 —Increase in height of *Atropa belladonna*

DISCUSSION

Characteristic "gibberellin-effects" were demonstrated in belladonna subjected in 1957 to three-spray treatments of G. A. in concentrations of 100 and 1,000 p p. m. (1) Among the specific effects reported for the treated plants were: increased stem elongation which resulted in a two- to three-fold increase in height; a spindly and vine-like type of growth; leaves which were slightly chlorotic, narrower, and thinner; more rapid onset of flowering, significant decreases in leaves and tops and root dry weights accompanied by significant increases in stem weights; no significant changes in total dry weights Also, significant decreases in the concentration of alkaloids were noted in the plant organs The total alkaloid content of the treated plants amounted to about one-half that of the controls

Belladonna receiving a treatment with 100 p p m of G. A. in 1957 generally did not demonstrate the aforementioned "gibberellin-effects" in their second-year growth in 1958. In several instances the re-

TABLE I.—WEIGHTS OF SECOND-YEAR GROWTH OF *Atropa belladonna* (AV./PLANT/GROUP)

Treatment	Total Weight: Fresh Gm	Total Weight: Dry, Gm	Total Weight: Control Dry Wt, %	Leaves and Tops: Fresh, Gm	Leaves and Tops: Dry, Gm	Leaves and Tops: Control Dry Wt %	Stems: Fresh, Gm	Stems: Dry Gm	Stems: Control Dry Wt, %	Roots: Fresh, Gm	Roots: Dry, Gm	Roots: Control Dry Wt, %
Control												
First harvest	247 2	33 3		123 2	16 8		123 0	16 5				
Second harvest	581 3	104 5		257 8	39 4		162 5	24 9		160 9	41 2	
Total	828 5	137 8		381 0	56 2		286 5	10 4		160 9	41 2	
100 p p m G A												
First harvest	228 2	30 7	92 2	112 4	15 5	92 3	115 8	15 2	92 1			
Second harvest	540 8	95 4	91 4	254 1	36 1	90 6	136 9	21 5	90 0	119 8	37 8	91 7
Total	769 0	126 1	91 5	366 5	51 6	91 8	252 7	36 7	90 8	119 8	37 8	91 7
1 000 p p m G A												
First harvest	166 5	20 7	62 2	85 1	11 1	66 1	81 0	9 6	58 2			
Second harvest	318 4	48 5	46 4	157 0	20 3	50 5	82 4	10 7	41 8	79 1	17 5	42 5
Total	484 9	69 2	50 2	242 4	31 4	55 9	163 4	20 3	50 2	79 1	17 5	42 5

TABLE II.—ANALYSIS OF ALKALOIDS[a] IN BELLADONNA

Treatment	Total Alkaloids, mg./Gm. Leaves and Tops	Stems	Roots	Total Alkaloid Content,[b] mg. Per Plant	Leaves and Tops	Stems	Roots
Control							
First crop	3.44	3.80		120.5	57.8	62.7	
Second crop	4.20	1.98	3.47	355.8	165.5	47.3	143.0
Total crop	3.97[c]	2.72[c]	3.47	476.3	223.2	110.0	143.0
100 p. p. m. G. A.							
First crop	3.59	3.59		109.0	54.3	54.7	
Second crop	4.22	2.13	3.33	324.0	152.3	45.8	125.9
Total crop	4.00[c]	2.74[c]	3.33	433.0	206.6	100.5	125.9
1,000 p. p. m. G. A.							
First crop	3.53	4.87		86.0	39.2	46.8	
Second crop	4.19	3.05	3.54	179.7	85.1	32.6	62.0
Total crop	3.96[c]	3.91[c]	3.54	265.7	124.3	79.4	62.0

[a] Total alkaloids calculated as hyoscyamine. [b] Alkaloid content for plant parts calculated from dry wt. and alkaloid analyses data; total plant alkaloid = leaves and tops + stems + roots. [c] Obtained by calculation.

verse effect was noted. Compared with controls, the treated plants were shorter, had decreased rather than increased stem weights, the onset of flowering was slower, and the concentration of alkaloids in the plant parts and the total alkaloid content did not change significantly. In general, this group of plants closely resembled the controls.

A few significant differences were noted, however, in the group previously treated with the higher concentration (1,000 p. p. m.) of G. A. In addition to the aforementioned reversals, significant decreases in total dry weight and in the weights of the plant organs were noted. The total dry weight of the second year crop of this group was about 50% of that of the controls. At each harvest the treated plants attained a height of about 85% of that of the controls. The concentration of alkaloids in the leaves and roots was about the same as the controls. However, the quantity of total alkaloids in the stems of the second year crop was about 44% greater than that of the controls. This effect was the reverse of that observed in the first year crop, since in that case, a 57% decrease was observed. It should be pointed out that following treatment in 1957 the increased stem elongation resulted in significant increases in plant height and stem dry weights. The second year growth demonstrated the reverse trend—shorter plants and significantly decreased stem weights. It is possible that in this case there was less stem area to dilute a fixed amount of alkaloid biosynthesized by the roots, or, since the active growth shown by the stem tissues in 1957 did not occur in the second year crop, there was not a diversion of nitrogenous material which might be used for stem elongation and growth, but, rather was used for alkaloid biosynthesis. It was also observed during both harvests of the 1958 crop that the stems of this group were less woody and the plants had matured more slowly than those of the other groups. The above factors might possibly explain the increased concentration of alkaloids in the stems.

The most striking change in the second year crop was that the plants previously treated with the higher concentration of G. A. had a total alkaloid content about one-half that of the controls. This was due primarily to approximately a 50% decrease in dry weight of this group. It is true that the first year crop of this group demonstrated a similar trend but the 50% decrease in total alkaloid content, in this case, was due primarily to decreases in the concentration of alkaloids in the plant organs. It also appeared that the treatment with the stronger concentration of G. A. was toxic. This is attested to by the fact that the second year growth was significantly less than that for the untreated plants and that two of the eighteen plants did not survive the winter whereas all of the controls did. These factors and the observation that the plants of this group did not appear as healthy as the controls during the growing season indicate that the higher concentration of G. A. had a toxic effect on the plants. This confirms the observation previously reported (1).

Some very interesting observations were made in comparing the second crop obtained in 1958 with the first crop harvested in the same year. The weights of the shoots of the second crop were about twice as large as the corresponding weights of the first crop. This increase was due to several factors: a more favorable growing season (summer and early fall), fertilization of the plot after the first harvest, and the fact that the plants were allowed to grow to a later stage of maturity. The concentration of alkaloids in the leaves and tops of the three groups was significantly higher in the second crop. This was unexpected since the first group was harvested at the flowering stage whereas the second crop was harvested at the fruiting stage, i. e., many of the plants had green berries. Several workers (3–6), have reported a higher content of alkaloids at the flowering stage than at either an earlier or later stage of development. Evans and Partridge (6) have reported that the concentration of alkaloids is the highest during flowering but declines during fruiting for both the first and second year growth of belladonna. Our results are not in agreement with these reports. However, it is possible that collecting two harvests from the second year growth may have been a factor. The advanced maturity of the stems as indicated by their woody character explains the significant decrease in the concentration of alkaloids in the stems of each group.

When the total 1958 crop was compared with the single 1957 crop the following differences were found: a 28 fold increase in the total dry weights of the control plants and those treated with 100 p. p. m. of G. A. compared with a 14 fold increase in the plants treated with the higher concentration of G. A. The concentration of alkaloids in the controls increased

about 18% in the leaves and tops and decreased 36% in the stems and 20% in the roots; the corresponding figures for the plants previously treated with 100 p. p. m. of G. A. were approximately a 108% increase, a 22% increase, and a 7% decrease; those for the plants treated with 1,000 p. p. m. of G. A. were increases of about 113%, 112%, and 7%, respectively.

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The Infection of Rye Callus With *Claviceps purpurea**

By DAVID P. CAREW and ARTHUR E. SCHWARTING†

The methods and results of inoculating callus cultures of rye (*Secale cereale*) with ergot (*Claviceps purpurea*) are described. The fungus grows intercellularly and invades some cells, but does not produce alkaloids.

THE ARTIFICIAL CULTURE of *Claviceps purpurea* (Fries) Tulasne for successful alkaloid production is a problem which has been widely investigated. The majority of these investigations have been concerned with the growth of *C. purpurea* on synthetic media to obtain an understanding of the factors concerned with alkaloid formation.

Recently the present authors reported the successful production and maintenance of a callus culture of rye, *Secale cereale L.*, on artificial media (1). Since rye is a natural host for ergot, this tissue was used in this study to investigate certain aspects of the host-parasite relationship.

EXPERIMENTAL

The host tissue consisted of a mass of callus cells which had originally been produced from fifteen to twenty-day-old embryos of rye (*Secale cereale*) growing on Heller's modified media (1) solidified with 1.0% agar. The tissue cultures had been maintained and transplanted to fresh media at approximately twenty-five day intervals for more than a year.

The rye callus was transplanted to agar plugs in 25 mm. x 150 mm. Pyrex culture tubes. A sterile, Pyrex glass cylinder, approximately 1.25 cm. long and 15 mm. in diameter, was inserted into each tube, in a position surrounding the callus and was made to penetrate the agar surface. Growth continued for approximately fifteen days, until the lateral callus growth covered the agar surface within the cylinder. This design prevented the inoculum, when introduced, from coming into direct contact with the agar.

The inoculum was prepared as follows: mycelial growth of *Claviceps purpurea* strain RCNA2, obtained from mature rye sclerotia and maintained on nutrient agar, was used to inoculate a liquid medium consisting of casein hydrolysate, 10.0; mannitol, 20.0; KH_2PO_4, 2.5; and $MgSO_4 \cdot 7H_2O$, 1.25 Gm./L. distilled water. The inoculated liquid media was grown as a submerged culture for eight days to provide inoculum for the callus growth.

The inoculum, 0.4 ml., was delivered to the surface of the callus growth. The tubes were plugged with cotton, capped with aluminum foil, and incubated in the dark, in a vertical position at 25°.

Infected callus was removed from the tubes at various intervals during forty days. In each case, the tissue was fixed in formalin-acetic acid-alcohol, dehydrated, and embedded in paraffin. The embedded tissue was sectioned with a rotary microtome and the sections stained in safranin-fast green. Infected tissue, of equivalent age, was dried, defatted, extracted in the usual manner, and qualitatively tested with Ehrlich's reagent. After forty days a quantity of tissue, obtained by pooling the infected tissue of a number of tubes, was dried at 32° in a circulating-air oven. The product was extracted and assayed (2) for total alkaloids.

EXPERIMENTAL RESULTS

Microscopic examination of the inoculated callus revealed the early intracellular growth of ergot mycelium. The penetration of occasional cells occurred within twenty-four to forty-eight hours. The arrow in Fig. 1 is directed to a penetrated cell wall. Extensive intercellular growth was always observed and can be seen among the rye callus cells in the figure. The majority of mycelial growth was superficial to the callus structure.

Extracts of the infected callus, when tested quali-

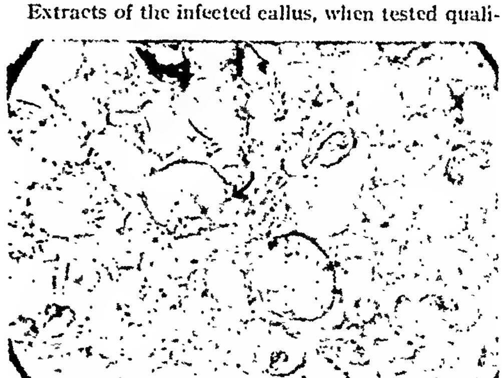

Fig. 1.—Rye callus infected with *C. purpurea*. Arrow indicates a single cell penetrated by the fungus.

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tatively with Ehrlich's reagent, consistently gave negative tests for alkaloids. The larger quantity of tissue, 2.813 Gm., dry weight, contained no alkaloids upon quantitative assay.

SUMMARY AND CONCLUSIONS

A callus culture of rye readily supports the growth of ergot and there is definite cellular penetration by the fungus. While callus is a satisfactory host for the fungus, there is no evidence of alkaloid formation during prolonged host-parasite association.

The early penetration and the intracellular invasion of rye callus observed here is analogous to the natural infection phenomenon (3, 4). The results, however, are contrary, in part, to those (5) reported on the growth of ergot on intact and punctured wheat root and rye embryo cultures.

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The Reduction of Hydrolysis Time and Tryptophan Destruction in the Preparation of Casein Hydrolysates*

By VINCENT S. VENTURELLA†, ROBERT W. SAGER, and JOSEPH A. BIANCULLI

A method was developed for hydrolyzing casein using a procedure which reduces the time requirements of enzymatic protein hydrolysis and minimizes the tryptophan destruction encountered in acid protein hydrolysis. Hydrolysates prepared by this method in twenty-four hours or less are comparable to enzymatic hydrolysates requiring one hundred hours. The method involves the treatment of partial enzymatic hydrolysates with diluted acids.

THE IMPORTANCE of protein hydrolysates in the treatment of certain deficiency symptoms and related conditions is well known. Such conditions as impaired digestion or absorption, poor assimilation because of liver damage, febral states, leukemia, hemorrhage, severe operations, burns, fractures, or shock usually result in an increased loss of amino acids and proteins (1, 2).

The parenteral administration of protein hydrolysates to maintain positive nitrogen balance is indicated in patients who are debilitated or are allergic to the administration of whole protein. It is essential that these preparations meet the usual requirements for intravenous preparations, and they should not contain high molecular weight peptides (2).

If a protein such as casein could be partially hydrolyzed by enzymatic methods to a low molecular weight polypeptide stage, it would seem possible that treatment with diluted acid should hydrolyze these polypeptide chains to even smaller peptides in a shorter period of time than would be needed by using enzymes alone. The amount of tryptophan destruction should also be less than that ordinarily observed with the use of strong acid alone.

EXPERIMENTAL

The moisture content of acid-washed casein,[1] determined in duplicate, was found to be 7.78%. All analytical results are calculated on the basis of dry casein.

Analytical Procedures.—*Total Nitrogen*.—The total nitrogen present in the casein sample was determined using a modification of the semimicro Kjeldahl method described in the United States Pharmacopeia (3). The method was modified by using 0.5 Gm. each of copper sulfate and potassium sulfate, together with a Hengar selenized granule. The average total nitrogen content of the casein sample was 15.41%.

Amino Nitrogen.—The amino nitrogen content of whole casein and of samples of hydrolysates was determined using a modification of the Sorenson formol titration described by Sahyun (4). A line operated pH meter, Beckman Model H, No. 487 was used to determine the necessary end points. Sodium hydroxide solution, 0.2 N, was used as the base. The amount of amino nitrogen was calculated using the following equation: $(A - B) \times 2.8$ mg. = mg. of amino nitrogen, where A = volume of sodium hydroxide solution for sample, and B = volume of sodium hydroxide solution used for the blank.

Using the average per cent total nitrogen present in the casein samples, the theoretical amount of amino nitrogen present at complete hydrolysis was

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† George A. Kelly, Sr., Fellow, 1954–1955.

[1] Fisher Scientific Co., No. C-200.

calculated. This calculation was made from data obtained in an experiment in which 20 Gm. of casein was digested for twenty-six hours in refluxing 6 N sulfuric acid. A sample of casein so treated was found to contain 12.59% amino nitrogen. A comparison of amino nitrogen and total nitrogen showed that the percentage ratio of amino nitrogen to total nitrogen was 81.7% This is the percentage of total nitrogen present as amino nitrogen.

The experimental conditions (5, 6), the results of this amino nitrogen analysis, and the nitrogen ratio obtained (7) were consistent with literature reports. Further evidence that the casein had been completely hydrolyzed was obtained by digesting the casein under identical conditions for two more hours. The amino nitrogen content of the hydrolysate did not change.

Colorimetric Determination of Tryptophan.—The amount of tryptophan present in the samples, presumably in the free or simple peptide stage, was determined colorimetrically by an adaption of Winkler's modification of the Hopkins-Cole method (8). The glyoxylic acid employed in the color reagent was prepared by reduction of oxalic acid with magnesium (8). In each case throughout the study, a 0.3-ml. sample of the hydrolysis mixture was mixed with 1.5 ml. of the color reagent. The volume of the mixture in each sample tube was adjusted to 9.5 ml. with deionized water prior to the addition of 15 ml. of sulfuric acid. The treated samples were placed in 2.5-cm. diameter cuvettes and the transmittance was determined with a Fisher electrophotometer, using a 525 mμ filter. Deionized water was used as the blank.

To ascertain the validity of the Lambert-Beer law in this determination, known concentrations of L-tryptophan solutions were determined colorimetrically. The results showed good conformity to the law by the production of a straight line plot when using concentration in μg. as ordinate and log I/I_0 as abscissa. Thus it was possible to calculate concentration of unknowns by means of the following equation, which is a form of the Lambert-Beer law (9): $\log I/I_0 = -a_s bc$, where a_s is the absorbance index, b is the diameter of the cuvette, and c is the concentration. The per cent transmission values of various concentrations of a series of samples of L-tryptophan appear in Table I. For the series of known values plotted, a value of $5.57 \times 10^{-4}\ \mu\text{g.}^{-1}\ \text{cm.}^{-1}$ was calculated for the absorbance index.

TABLE I.—TRANSMISSION OF LIGHT OF THE COLORED REACTION PRODUCT OF CRYSTALLINE L-TRYPTOPHAN AND GLYOXYLIC ACID AT 525 mμ

L-Tryptophan, μg.	Transmission, %	Log I/I_0
28.8	88.6	−0.054
57.5	85.0	−0.070
115.0	72.9	−0.138
201.3	53.5	−0.272
287.5	40.7	−0.390
373.8	31.5	−0.503

Electrophoretic Tryptophan Determination.—The concentration of tryptophan was also determined for a number of the hydrolysates by the use of paper electrophoresis as a check on the colorimetric method. This method was possible because an amino acid in solution at a pH other than at its isoelectric point will migrate under the influence of an electric force. Paper electrophoresis makes use of this property (10, 11).

Two series of experiments were performed using a Reco Model E-800-2 migration chamber and power supply. This instrument operates on the horizontal "open-strip" method (11). The position of the amino acid spot was determined by developing with 0.1% ninhydrin in 95% ethanol in the usual manner (12). The color density curves of the samples of crystalline L-tryptophan and tryptophan isolated from the hydrolysate samples, according to the method of Cox and King (13), were determined using a Photovolt densitometer, model 425. To compare the amount of crystalline L-tryptophan against the amount of tryptophan isolated by this method, it was necessary to compare the area under the standard curve with the area under the curve obtained from the unknowns. This was accomplished by applying the trapezoidal rule (14) to a plot of the absorbance as abscissa against the migration distance as ordinate.

The hydrolysate samples were placed on Whatman No. 3, $1^1/_2$-inch strips as the support. Buffers of 5 N acetic acid (15), pH 1.8, and 0.1 M phosphate, pH 3.5, were used in two successive experiments. When 5 N acetic acid was used, a current of 0.33 ma. at a potential of 500 v. was applied for two hours. This produced a migration of 2.9 cm. In the experiment employing the phosphate buffer, a potential of 450 v. was applied for the same period of time using a current of 3.5 ma. The migration in this case was 3.9 cm. The migration in both cases was toward the cathode as shown in Fig. 1.

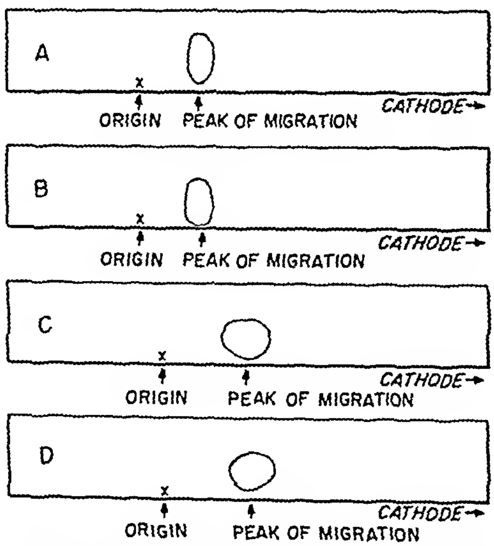

Fig. 1.—Electrophoretic migration of L-tryptophan. Strips A and B using 5 N acetic acid, pH 1.8. Strips C and D using 0.1 M dibasic sodium phosphate-phosphoric acid, pH 3.5. Strips A and C represent crystalline material. Strips B and D represent isolated material.

The results of a few comparisons appear in Table IV. The results obtained electrophoretically when compared to the colorimetric results show good correlation of the two methods.

Hydrolytic Methods.—*Enzymic Hydrolysis.*—

The enzymatic digestion of casein was studied in order to determine the relative rate of release of amino nitrogen. This was done because it was the partial enzymatic hydrolysates showing the most uniform release of amino nitrogen that would be chosen for further study.

One hundred grams of casein, suspended in 690 ml. of deionized water, was mixed with 6 Gm. of U. S. P. pancreatin. The pH was adjusted to 8.5 with 4 *N* sodium hydroxide solution. The suspension was stirred for one hour, and then adjusted to pH 7.9–8.0. It was then diluted to 1,000 ml., toluene was added to just cover the top of the suspension, and then the mixture was incubated at 37°.

Ten milliliter portions of the hydrolysates were periodically removed, and after each removal the pH of the suspension was adjusted to 8.0. The amino nitrogen content of each portion was determined. The rate of enzymatic hydrolysis was thus studied by plotting the mg. of amino nitrogen released against time of hydrolysis. The results of the analyses are tabulated in Table II and shown graphically in Fig. 2.

TABLE II.—ENZYMATIC HYDROLYSIS

Hours of Digestion	Amino Nitrogen Released[a]	Nitrogen Ratio[b]
0.0	14.8	0.117
4.5	28.4	0.225
20.0	35.1	0.280
26.3	38.1	0.301
29.5	38.6	0.307
51.3	14.0	0.350
99.3	44.7	0.353

[a] Mg. per Gm. of dry casein.
[b] Based upon the ratio of amino nitrogen/total nitrogen in complete acid hydrolysis.

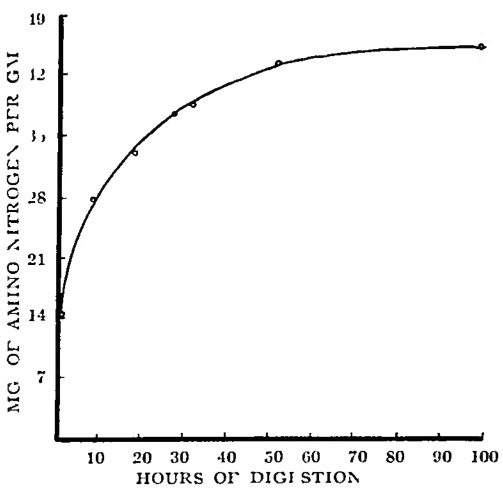

Fig. 2.—Rate of amino nitrogen release in the pancreatic digestion of casein.

The hydrolysates chosen as the ones presenting the most uniform release of amino nitrogen were thirteen to twenty-four hour hydrolysates. These hydrolysates were the ones chosen for the modified hydrolytic procedure.

Acidic Hydrolysis.—A casein sample was completely digested with 6 *N* sulfuric acid as previously described, to determine the amino nitrogen present on complete hydrolysis and to determine, colorimetrically, the amount of tryptophan present. In this experiment, a sample of the hydrolysate itself was used as the blank since the hydrolysate was slightly discolored. This experiment yielded 12.59% amino nitrogen based upon the dry weight of casein. The sample contained 0.06% tryptophan.

Modified Enzymatic and Acidic Hydrolysis.—A modified hydrolytic procedure was then used with a series of enzymatic hydrolysates, the time of hydrolysis falling in the range of uniform release of amino nitrogen. At the end of thirteen to twenty-four hours of enzymatic hydrolysis, the mixture was heated on a water bath at 80° for ten minutes to deactivate the enzyme. After the suspension cooled, 10 Gm. of charcoal was added to partially decolorize the hydrolysate and it was filtered at reduced pressure through a Buchner funnel.

A sample of the filtrate so obtained was analyzed for amino nitrogen and tryptophan. Five hundred milliliters of the filtrate was then mixed with 500 ml. of the appropriate diluted sulfuric acid solution and refluxed at 103° for the prescribed interval of time. At the end of this time, the solution was cooled and the amino nitrogen content and tryptophan content again determined.

A summary of the amino nitrogen determinations and tryptophan analyses, before and after acid treatment, with nitrogen ratio, appears in Table III.

TABLE III.—RESULTS OF THE MODIFIED HYDROLYTIC PROCEDURES

Hours of Hydrolysis Enzyme	Hours of Hydrolysis Acid	Molarity of Acid	Amino Nitrogen[a]	Tryptophan %	Nitrogen Ratio
13			15.0	0.54	0.119
13	2	0.2	17.7	0.66	0.141
13	3	0.2	24.4	0.52	0.191
13	2	0.3	18.2	0.55	0.115
16			20.5	0.59	0.163
16	2	0.2	25.8	0.94	0.205
16	3	0.2	26.0	0.92	0.207
16	2	0.3	26.0	0.88	0.207
17			34.2	0.98	0.272
17	2	0.2	46.0	1.21	0.366
17	2	0.1	43.3	1.05	0.341
17	2	0.3	50.9	0.92	0.405
20			31.5	0.96	0.250
20	2	0.2	40.8	1.26	0.324
22			34.6	0.95	0.275
22	2	0.2	44.6	1.36	0.352
24			34.9	0.79	0.278
24	2	0.2	46.7	0.79	0.372

[a] Mg. per Gm. of dry casein.

TABLE IV.—COMPARISON OF % TRYPTOPHAN OBTAINED COLORIMETRICALLY AND ELECTROPHORETICALLY

Hours of Hydrolysis Enzyme	Hours of Hydrolysis Acid	Acid Molarity	Tryptophan % Colorimetric	Tryptophan % Electrophoretic
16	2	0.2	0.94	0.87
17	2	0.2	1.21	1.09
20	2	0.2	1.26	1.15
24	2	0.2	0.79	0.71

Av. % Difference 0.08
Av. % Deviation—7.60

SUMMARY AND CONCLUSIONS

The results of the method described to decrease both the time of hydrolysis and the destruction of tryptophan in preparing casein hydrolysates indicates the potential value of the method. The data tabulated in Table III suggest that hydrolysis with pancreatin for seventeen to twenty-two hours followed by refluxing with 0.2 *M* sulfuric acid for two hours, results in a product containing about 45 mg. of amino nitrogen per Gm. of casein and about 1.3 per cent tryptophan. These products represent a nitrogen ratio of about 0.36.

The desirability of this method arises from the saving in time because the degree of hydrolysis obtained, although no greater than commercial enzymatic products, was attained in seventeen to twenty-two hours, whereas it was noted that one hundred hours digestion with pancreatin alone is necessary to attain the same result. Commercial acid hydrolysates usually exhibit a greater degree of hydrolysis with a concurrently greater destruction of tryptophan.

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Substituted Salicylanilides With Antimicrobial Activity*

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Eighteen new halonitrosalicylanilides have been prepared. Of these the salicylanilides of 3-nitro- and 5-nitrosalicylic acid, halogenated by one, two, or three halogens of various kinds in the aniline nuclei, have been shown to possess a high degree of bacteriostatic and fungistatic activity.

SALICYLANILIDE, a widely-used commercial fungistat, was first recognized by Fargher and co workers (1) to be an excellent textile preservative. Derivatives of salicylanilide have been finding increasing mention in the literature Thus, apparently in trying to widen the range of antimicrobial activity, interesting compounds such as those wherein the salicylanilide moiety and sulfonamide moieties have been united (2–5) or an anilide made from *p*-aminosalicylic acid (6–12) have been examined. However, the former compounds were not reported to have exceptionally better antimicrobial properties than salicylanilide or the sulfonamides alone, and the latter were not reported as being superior to *p*-aminosalicylic acid as tuberculostats. Recently, polyhalogenated salicylanilides have received considerable attention (13–15), it being reported that particularly 5-bromo-4′-chlorosalicylanilide has a high potency and wide range of antimicrobial action.

Salicylanilide and substituted salicylanilides have been previously prepared in low yields by the direct condensation of salicylic acid, or the substituted acid with aniline, or a substitution derivative of aniline (16). Various condensation agents such as aluminum anilide (17), phosphorus trichloride (11, 14–16, 18, 19), and tetralkyl pyrophosphites or dihalophosphites (20) have been used to increase the efficiency of the reaction greatly. These compounds have also been prepared in good yields by the reaction of the phenyl or methyl esters of the particular salicylic acid with the appropriate aniline (14, 21). Salicoyl chloride and substituted salicoyl chlorides have been reacted with various anilines to give the salicylanilides (14, 22). The acid chlorides have been prepared by classical means without the necessity of blocking the *ortho* hydroxyl group (23, 24). Of a number of other methods which are less orthodox and generally less efficient, two are particularly interesting.

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Thus, *ortho*-carbamyl derivatives of diphenyl ethers have been rearranged to salicylanilides (25). Further, the sodium derivative of the phenyl ester of phenylcarbamic acid has been rearranged to give a 20 per cent yield of salicylanilide (26).

Substituted salicylanilides have also been prepared by the direct action of reagents upon salicylanilide. Thus, nitration (16), chlorination with sulfuryl chloride (27), sulfonation (28), and bromination (14, 29, 30) of salicylanilide or substituted salicylanilides have been studied.

The selection of the type of compounds prepared in the present work was partly based on results with nitro- (31, 32) and halo-2-furanacrylanilides (32), which exhibit bacteriostatic activity, and 4′-chloro-5-nitro-2-furanacrylanilide (33), which exhibits anthelmintic activity. Thus it seemed that halonitrosalicylanilides, which would have a number of chemical features common to some of the above mentioned 2-furanacrylanilides and also possess the salicylanilide moiety of known antifungal activity, might exhibit broader antimicrobial action than either of the above materials. Indeed, when 4′-chloro-5-nitrosalicylanilide was prepared and tested, this was found to be the case. Therefore, seventeen analogs of this compound were prepared to determine the effects, due to changes in the positions and the amounts of various groups present or due to the kinds of halogen substituent, upon antimicrobial activity.

Experiments have been carried out to prepare these compounds by three methods. Reaction of the respective dinitro- or nitrosalicoyl chlorides with an excess of the halogenated aniline in benzene was found to be a satisfactory method which gave the halonitrosalicylanilides in 71 to 93 per cent yields. Most of the salicylanilides in the present work were made by this method. However, interaction of the acid, the aniline, and phosphorus trichloride in benzene was also found to be useful. In a third method, a Schotten-Baumann reaction between the acid chloride and the aniline in the presence of aqueous sodium hydroxide was performed to give, however, low yields of the desired anilides highly contaminated by the acids. The compounds prepared and their physical properties are given in Table I. The known materials 4′-chlorosalicylanilide and 3-nitrosalicylanilide were also prepared, using the Schotten-Baumann method, for comparison with the other anilides in the biological testing.

The 3- and 5-nitrosalicylic acids used in this work were obtained by a low temperature (0°) nitration of salicylic acid which yielded an isomeric mixture of about 3:1 of the 5- and 3-nitro acids. Separation was effected by taking advantage of the difference in solubility of the potassium salts of these acids (34).

The acid chlorides were than prepared directly from the above acids upon refluxing with thionyl chloride in benzene in the presence of a small amount of anhydrous aluminum chloride. Three hours were required for the completion of this reaction with the 3-nitrosalicylic acid. However, sixteen hours were required for the 5-nitro- and 3,5-dinitrosalicylic acids to form the chlorides. The aluminum chloride catalyst was important for the latter two acids since without it the reaction was not complete in sixteen hours. 5-Nitrosalicoyl chloride had not been previously prepared.

The halonitrosalicylanilides prepared in this work are insoluble in water. However, because of the presence of the phenolic group whose acidity is enhanced by the nitro group on the same ring, aqueous solutions of about 0.05 per cent could be prepared for antimicrobial tests by adding an equivalent of suitable base such as sodium hydroxide to the water.

PHARMACOLOGICAL ACTIVITY

Antibacterial screening by the agar cup-plate method among all of the compounds listed in Table I indicated that the halo-3,5-dinitrosalicylanilides had little or no activity against the bacteria referred to below. However, the halo-3-nitro- and halo-5-nitrosalicylanilides are potent antimicrobials against a number of bacteria and fungi. Preliminary comparison tests by the agar cup-plate method among the salicylanilides indicated that all of the halo-3-nitrosalicylanilides had very similar potencies and specificities of action regardless of the number, kind, or position of the halogens in the aniline ring. This was also found to be true for the halo-5-nitrosalicylanilides. Therefore, 4′-chloro-3-nitrosalicylanilide (I) and 4′-chloro-5-nitrosalicylanilide (II) were selected as representatives of these two groups for further testing. The influence of the various substituent groups particularly against *Micrococcus pyogenes* var. *aureus* is illustrated in Table II. I also showed a threshold inhibition at 18 mcg./cc. against *Micrococcus flavus* and II showed inhibition at 10 mcg./cc. against *Alcaligenes faecalis*.

These results indicate that both the nitro and halogen groups confer antibacterial activity upon the salicylanilide parent molecule, which when unsubstituted exhibits very little antibacterial action. The absence of much activity among either the halogenated anilines or the nitrosalicylic acids indicates that this modification in activity of salicylanilide is not due simply to the fact that the molecule is now a combination of an aryl nitrophenol and a halogenated aniline. Instead, it appears to be a more complex gross effect requiring, particularly, the anilide linkage to complete the

TABLE I.—HALONITROSALICYLANILIDES

Compound	M. p.,[a] °C	Formula	Analysis: Carbon, %, Calcd	Carbon, %, Found	Hydrogen, %, Calcd	Hydrogen, %, Found
4′-Fluoro-3-nitrosalicylanilide	141.5–142	$C_{13}H_9FN_2O_4$	56.53	57.19	3.20	3.35
2′-Chloro-3-nitrosalicylanilide	171.5–172.5	$C_{13}H_9ClN_2O_4$	53.34	53.42	3.09	3.16
3′-Chloro-3-nitrosalicylanilide	152.5–153.5	$C_{13}H_9ClN_2O_4$	53.34	53.36	3.09	3.06
4′-Chloro-3-nitrosalicylanilide	154–156	$C_{13}H_9ClN_2O_4$	53.34	53.10	3.09	2.99
4′-Bromo-3-nitrosalicylanilide	158–158.5	$C_{13}H_9BrN_2O_4$	46.31	46.36	2.69	2.69
4′-Iodo-3-nitrosalicylanilide	175–178	$C_{13}H_9IN_2O_4$	40.64	41.26	2.36	2.67
2′,4′-Dichloro-3-nitrosalicylanilide	241	$C_{13}H_8Cl_2N_2O_4$	47.71	47.42	2.46	2.28
4′-Fluoro-5-nitrosalicylanilide	240	$C_{13}H_9FN_2O_4$	56.53	58.32	3.20	3.37
2′-Chloro-5-nitrosalicylanilide	200–203	$C_{13}H_9ClN_2O_4$	53.34	53.11	3.09	3.01
3′-Chloro-5-nitrosalicylanilide	230	$C_{13}H_9ClN_2O_4$	53.34	53.55	3.09	3.07
4′-Chloro-5-nitrosalicylanilide	252.5	$C_{13}H_9ClN_2O_4$	53.34	53.51	3.09	3.09
4′-Bromo-5-nitrosalicylanilide	242–245	$C_{12}H_9BrN_2O_4$	46.31	46.15	2.69	2.49
4′-Iodo-5-nitrosalicylanilide	261–264	$C_{13}H_9IN_2O_4$	40.64	41.05	2.36	2.56
2′,4′-Dichloro-5-nitrosalicylanilide	261–262	$C_{13}H_8Cl_2N_2O_4$	47.71	47.65	2.46	2.20
2′,4′,6′-Trichloro-5-nitrosalicylanilide	233.5–234	$C_{13}H_7Cl_3N_2O_4$	43.28	43.09	1.95	1.81
2′-Chloro-3,5-dinitrosalicylanilide	216.5–218	$C_{13}H_8ClN_3O_6$	46.24	45.96	2.39	2.28
3′-Chloro-3,5-dinitrosalicylanilide	211–212	$C_{13}H_8ClN_3O_6$	46.24	47.67	2.39	2.81
4′-Bromo-3,5-dinitrosalicylanilide	227	$C_{11}H_8BrN_3O_6$	40.86	41.09	2.11	2.46

[a] Corrected.

TABLE II.—ANTIBACTERIAL ACTIVITIES

Compound	Bacterial Inhibition Threshold Concentrations mcg./cc. *S. aureus*[a]	*B. subtilis*[b]	*E. coli*[c]
5-Nitrosalicylic acid	500[d]	.	.
3,5-Dinitrosalicylic acid	500	.	
4-Chloroaniline hydrochloride	500		
2,4-Dichloroaniline hydrochloride	500[d]		.
Salicylanilide	1000[d]	1,000[d]	1,000[d]
4′-Chlorosalicylanilide	10	.	.
3-Nitrosalicylanilide	10	.	.
4′-Chloro-3-nitrosalicylanilide	1	1	1,000[d]
4′-Chloro-5-nitrosalicylanilide	1	0.3	1,000[d]

[a] *Micrococcus pyogenes*, var. *aureus*.
[b] *Bacillus subtilis*.
[c] *Escherichia coli*.
[d] No inhibition.

TABLE III.—SERIAL BROTH DILUTION DETERMINATIONS

Organism	Inhibiting Dose, µg./cc. 4′-Chloro-3-nitrosalicylanilide	4′-Chloro-5-nitrosalicylanilide
Micrococcus pyogenes var. *aureus*	4–8	1–2
Bacillus subtilis	9–10	1–2
Pseudomonos aeruginosa	>200	>200
Escherichia coli	>200	>200
Alcaligenes faecalis	80–100	20–40
Proteus (species unknown)	>200	60–80

combination of moieties for the manifestation of markedly increased antibacterial activity.

I and II were compared and examined more thoroughly in broth serial dilution tests. The results of these tests are shown in Table III. From the screening tests and from the serial dilution tests it can be seen that the 5-nitro derivative is consistently more potent than the 3-nitro compound. Furthermore, it is evident that these halonitrosalicylanilides are consistently active against the Gram-positive organisms but are often inactive or less active against the Gram-negative organisms.

Upon testing the analogs against fungi a greater diversity of action was exhibited than against the bacteria. The 3,5-dinitrosalicylanilides again showed none or very little antimicrobial action. In these tests, I showed decidedly more inhibiting activity than II against *Trichophyton mentagrophytes, Candida albicans*, and *Penicillium luteum*. From general comparison tests carried out against these organisms, I and 3′-chloro-3-nitrosalicylanilide (III) exhibited considerably greater potencies against the above organisms than any of the other compounds. Inhibition tests at 286 p. p. m. in agar were carried out. At this level I and III were able to inhibit all growth of the *T. mentagrophytes* at fourteen days. However, for *P. luteum*, although complete inhibition was not obtained, these compounds caused the lowest radial growth rate. Further tests with III showed it to inhibit completely *E. floccosum* at 7.1 p. p. m. at twenty-five days. Upon comparing I and salicylanilide in a twenty-seven-day serial dilution test against *C. albicans*, both materials gave similar inhibition threshold values at 45 p. p. m.

The last results, together with some of the other numerical threshold values, indicate that although the nitro and halogen substituents confer antibacterial properties upon salicylanilide, they neither enhance nor lower its already high antifungal activity.

Preliminary toxicity studies indicate that the halo-3-nitrosalicylanilides are consistently at least several times more toxic to rats than the 5-nitro isomers. For example, I was found to have an intraperitoneal LD_{50} of 34 mg./Kg. in rats, while II had an LD_{50} of over 125 mg./Kg. in the same determinations.

EXPERIMENTAL

3- and 5-Nitrosalicylic Acids.—Nitrations in water, acetone, and acetic acid were carried out. However, the most satisfactory method was to combine a low temperature nitration procedure in sulfuric acid with the principle of the separation procedure of Meldrum and Hirve (34). Thus, 2,000 Gm. (14.5 mole) of salicylic acid was dissolved in 4 L. of concentrated sulfuric acid and the solution was cooled with dry ice–alcohol to 0°. Then with rapid stirring and continued cooling a precooled mixture of 916 ml. of concentrated nitric acid and 916 ml. of concentrated sulfuric acid was added, keeping the temperature below 7°. One hour was required for addition, after which time the mixture was poured into 24 L. of water, allowed to stand overnight, filtered by vacuum, washed, and dried at 70° for sixteen hours. A yield of 2,550 Gm. (96.0% yield) of the cream colored isomeric mixture was obtained. One hundred and sixty-three Gm. (0.89 mole) of the mixture was dissolved with stirring in 3,300 ml. of boiling water containing 114.0 Gm. of potassium carbonate and 15 Gm. of charcoal. The hot solution was filtered and allowed to stand at room temperature overnight. The orange crystals of the potassium salt of 3-nitrosalicylic acid which had precipitated were filtered by vacuum and washed three times with cold water. They were then dissolved in 1 L. of 90° water, the solution was filtered, and 60 ml. of 35.0% sulfuric acid was added to the hot filtrate with vigorous stirring to give an immediate precipitate of 3-nitrosalicylic acid. The solution was stirred and cooled to room temperature (about three hours) and the white solid that separated was removed by vacuum filtration and washed with water. This product was dried at 100° for sixteen hours to give 37.5 Gm. (22.5% yield) of 3-nitrosalicylic acid, m. p. 147.5–148° (lit. (35) m. p. 146–148°), which now had a canary yellow color. Upon acidifying the original filtrate and reducing its volume to one-fifth, 90 Gm. (55.0% yield) of 5-nitrosalicylic acid, m. p. 234–236° (lit. (35) m. p. 234°) was obtained upon filtering, washing, and drying.

3-Nitrosalicoyl Chloride.—Eighty Gm. (0.44 mole) of 3-nitrosalicylic acid, 52.0 ml. of thionyl chloride, and 320 ml. of benzene were refluxed for two hours. The solution was filtered and the solvents removed by evacuating at room temperature for sixteen hours. A yield of 82 6 Gm. (93.6%) of yellow crystals of 3-nitrosalicoyl chloride, m. p. 55.5–57.5° (lit. (36) m. p. 59–61°), was obtained.

Anal.—Calcd. for $C_7H_4ClNO_4$: C, 41.70; H, 2.00. Found: C, 43.62; H, 2.04.

When the acid chloride was heated to 60° during the removal of the solvents, hydrogen chloride was given off and the resultant product had a much poorer melting point.

5-Nitrosalicoyl Chloride.—This was prepared by essentially the same procedure used above, however, it was found that the reaction proceeded much more slowly. Thus, without a catalyst, at the end of sixteen hours, a considerable amount of the benzene-insoluble 5-nitrosalicylic acid had remained unconverted to the benzene-soluble acid chloride. When a small amount of anhydrous aluminum chloride was added to the reaction mixture, no insoluble material remained after sixteen hours of refluxing. Thus, 50.0 Gm. (0.27 mole) of 5-nitrosalicylic acid, 200 ml. of benzene, 30.0 ml. of redistilled thionyl chloride, and 0.1 Gm. of anhydrous aluminum chloride gave 49 7 Gm. (91.6% yield) of crude 5-nitrosalicoyl chloride. It was found that if heat was applied (60°) while removing last traces of solvents from the residue, hydrogen chloride was evolved and the syrupy residue would not solidify. If heat was not applied the crude acid chloride was obtained as a hard brittle glass. A portion of this glass was recrystallized from 1:1 petroleum ether–benzene to give, in the second crop, a pure sample of 5-nitrosalicoyl chloride, m. p. 78–80°, which was a cream colored amorphous solid.

Anal.—Calcd. for $C_7H_4ClNO_4$: C, 41.70; H, 2.00. Found: C, 42.59; H, 2.10.

3,5-Dinitrosalicoyl Chloride.—This material had been previously prepared with phosphorus pentachloride (24, 37). In the present work, the same method as was used above to prepare the 5-nitrosalicoyl chloride was applied. Here again sixteen hours of refluxing was necessary to complete the reaction with the aid of the anhydrous aluminum chloride catalyst. The crude 3,5-dinitrosalicoyl chloride was used in the preparation of the salicylanilides.

Halonitrosalicylanilides, 4′-Chloro-3-nitrosalicylanilide.—The method described below represents the general procedure used to prepare most of the halonitrosalicylanilides under discussion. When 3-nitrosalicoyl chloride was added to pyridine an exothermic reaction took place to give a pyridine-insoluble product. Thus, it was not possible to use pyridine as a solvent for anilide formation. A solution of 25 Gm (0.12 mole) of 3-nitrosalicoyl chloride in 200 ml. of benzene was added with shaking to 50 Gm. (0.39 mole) of *p*-chloroaniline in 150 ml. of benzene. An immediate yellow precipitate formed and the reaction mixture was allowed to stand overnight and vacuum filtered. The solid obtained was washed with small amounts of benzene, air dried, pulverized, and then stirred for thirty minutes in 100 ml. of 10% hydrochloric acid. The product was vacuum filtered, washed well with water, and dried at 90° for sixteen hours to give 28.5 Gm. (78.5% yield) of crude 4′-chloro-3-nitrosalicylanilide, m. p. 153–156°. This was crystallized from 600 ml. of ethanol to give 23.0 Gm. of yellow needles in a first crop and 3 6 Gm. more when the alcohol filtrate was reduced to one-sixth. Thus, a total of 26.6 Gm. of 4′-chloro-3-nitrosalicylanilide, m. p 154–156° was obtained.

Anal.—Calcd. for $C_{13}H_9ClN_2O_4$: C, 53.34; H, 3.09. Found: C, 53.10; H, 2.99.

When 2,4-dichloroaniline was reacted with the nitrosalicoyl chlorides, the rate of reaction was slower than with the monohaloanilines and a precipitate did not start to form until about one hour after mixing and did not fill the mixture until several hours later. With the monohaloanilines, precipitation occurred immediately and satisfactory yields could be obtained in about one hour after mixing. With 2,4,6-trichloroaniline and 3-nitrosalicoyl chloride, the anilide did not form until after five days. However, with 5-nitrosalicoyl chloride and the trichloroaniline the salicylanilide was obtained only after seven days standing. The halo-3-nitro and halo-3,5-dinitrosalicylanilides were crystallized from ethanol, while the

5-nitro compounds were crystallized from ethanol or ethanol-dimethylformamide.

4′-Chloro-5-nitrosalicylanilide by the "Phosphorus Trichloride Method."—Four grams (0.022 mole) of 5-nitrosalicylic acid, and 6 Gm. (0.047 mole) of *p*-chloroaniline were ground, intimately mixed, and placed into 35 ml. of benzene containing 1.5 ml. of phosphorus trichloride. The mixture was refluxed for sixteen hours, cooled, and filtered. The solid was crushed and suspended in 10% hydrochloric acid, filtered, and crystallized from ethanol to give 5.4 Gm. (83.1% yield) of 4′-chloro-5-nitrosalicylanilide, m. p. 249–250°. Upon admixture with 4′-chloro-5-nitrosalicylanilide prepared from the acid chloride (m. p. 252.5°) no depression of melting point occurred.

4′-Chlorosalicylanilide.—This was prepared by the Schotten-Baumann procedure. Salicoyl chloride was prepared according to the method of Wolffenstein (23) from thionyl chloride and salicylic acid. Thus 20.0 Gm. (0.13 mole) of salicoyl chloride in 20 ml. of benzene was added to a rapidly stirred mixture of 16.4 Gm. (0.13 mole) of *p*-chloroaniline and 15 Gm. of sodium hydroxide in 100 ml. of water. The acid chloride was added over a ten-minute period and the mixture was then stirred for thirty minutes. No solid had separated and thus the aqueous layer was acidified to pH 6 whereby 15.0 Gm. of solid that started to melt at 134° was obtained. Further acidification of the aqueous layer yielded salicylic acid, m. p. 157–159° (lit. (38) m. p. 157–159°). The solid first obtained was crystallized from ethanol to give 4.3 Gm. (13.4% yield) of 4′-chlorosalicylanilide, m. p. 169–170° (lit. (39) m. p. 168–169°).

3-Nitrosalicylanilide.—This was also prepared by the Schotten-Baumann method. In this preparation the product precipitated and was filtered directly from the reaction mixture. Acidification of the aqueous phase yielded only a small amount of additional material. Thus 4.0 Gm. (0.02 mole) of 3-nitrosalicoyl chloride and 1.9 Gm. (0.02 mole) of aniline in benzene with 1.5 Gm. of sodium hydroxide in 20 ml. of water gave 3.5 Gm. of insoluble precipitate. One gram of this material was suspended in boiling 4% sodium carbonate to remove any 3-nitrosalicylic acid and then suspended in 10% hydrochloric acid to remove unused aniline. The solid was filtered, washed, and recrystallized from ethanol to give 0.4 Gm. (25.0% yield for the total of original product assuming 40% recovery on purification) of 3-nitrosalicylanilide, m. p. 124–125° (lit (9) m p. 121–122°).

Antimicrobial Screening.—All of the compounds were tested in aqueous solutions made by dissolving, with warming, up to several hundred milligrams in 100 ml of water containing an equivalent or a slight excess of sodium hydroxide, to give solutions of pH 7 to 8 Antibacterial screening tests were done by the agar-cup plate method, placing the test compound solutions into the cups and seeding the agar with the organism under study (40). Values of threshold concentrations were approximated either by means of inoculated serial dilutions of the compounds or by plotting the logarithms of the concentrations against the zones of inhibition for several concentrations and extrapolating to the threshold values

The broth dilutions were prepared by incorporating appropriate graded amounts of the compounds in Difco Yeast Beef Broth which was maintained at a uniform concentration throughout each series. The tubes of the various series were inoculated with a loopful of inoculum from twenty-four hour cultures of the test organisms grown in the same medium. Incubation for forty-eight to seventy-two hours was usually sufficient although all were observed during a period of one week.

Fungistatic activity was determined by inoculating the test organism into a medium composed of a base of plain agar containing the test compound overlaid by a layer of potato dextrose agar. From periodic measurements of the radii of the colonies, the inverse of the radial growth rate in mm. per day was considered an index of the fungistatic efficacy of the test substance.

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Thermosterilized Acriflavine Emulsion Ointment[*]

By E. MENCZEL and S. GRUN

Four thermostable o/w emulsion bases were selected for the preparation of acriflavine ointments with which absorbent gauze was impregnated and steam sterilized for topical antisepsis. Bacteriostatic tests indicated that at 1 per cent w/w concentration of acriflavine effective antiseptic ointments were produced.

VARIOUS ACRIFLAVINE OINTMENTS are clinically used for topical antisepsis, without the complications of resistance encountered with antibiotics and other oral or parenteral bacteriostatic agents. Experimental data indicate that the bacteriostatic activity of acriflavine ointments depends also upon the ointment bases. Frank and Stark (1) tested the effectiveness of acriflavine[1] incorporated in a water-in-oil emulsion ointment base containing cholesterol; even at the concentration of 1.5 per cent w/w acriflavine, the growth of *Staphylococcus aureus* and *E. coli* was not inhibited. Owing to anionic cationic interaction, oil-in-water emulsion ointments of acriflavine with aqueous cream (3) B. P. base[2] could not have been prepared. Similarly, a sodium alginate base was not found suitable. Unguentum glycerini, a greaseless starch glycerite base official in the Swiss Pharmacopeia (4), did not hinder the antiseptic activity of the acriflavine incorporated, at the range of 1–1.5 per cent w/w concentration. This is in accordance with the prevailing concepts that some antiseptic ointments are more effective in water-miscible bases having a high water content (5). However, starch glycerite is apt to deteriorate rapidly; furthermore, water-miscible bases devoid of an oily phase are considered to be drying and not applicable to both dry thin and thick fatty skins (6). Eventually, a stable water-miscible emulsion base of high water content appears to be the formulation of choice for an acriflavine ointment. The clinical use of an acriflavine ointment in surgical theater and wards in the treatment of burns and other topical infections warrants absolute sterility to avoid reinfection by resistant microorganisms. Sterilization could be performed by one of the thermal procedures which, however, might prove deleterious to the thermolabile emulsion system. Benerito and Singleton (7) and Lambert, *et al.* (8), have reported studies on the preparation of thermostable emulsions for parenteral use which could be sterilized. Thermosterilizability will, therefore, feature as a prime factor in evaluating the o/w emulsion bases for an effective acriflavine ointment. As far as consistency is concerned, it should be softer than ordinary ointment bases and this might enable ready incorporation into a medicated dressing.

EXPERIMENTAL

Emulsifying Agents.—The preparation of o/w emulsion ointments of acriflavine precludes the use of anionic emulsifying agents. The selection has, therefore, to be made from the limited list of nonallergic, nonirritating cationic and nonionic emulsifying agent agents. As to the thermosterilizability of an o/w emulsion produced, a certain clue can be obtained by ascertaining the inversion temperature of a 1% solution of the emulsifying agent. The procedure of the test was exactly the same as that suggested by Benerito and Singleton (7). The nonionic emulsifying agents, polysorbate 80 and polyoxyl 30 stearate, were found to have high temperature inversion points[3] and eventually these emulsifying agents might produce thermostable o/w emulsion ointments. Preference was given to polyoxyl 30 stearate over its homolog containing 40 polyethylene groups (official in U. S. P. XV) owing to its high degree of diffusibility (9) which cannot be over-emphasized in this type of preparation. The nonionic emulsifying agents, trademarked in Germany as Cremophors, proved to have certain merits (10, 11) in emulsion technology but their thermostability could not have been deducted from the Benerito-Singleton test. A highly purified grade of egg lecithin (cationic emulsifying agent) deteriorated upon temperature rise when applying the Benerito-Singleton test.

Preparation of the o/w Emulsion Ointments, Stability Controls, and Their Physical Properties. Acriflavine o/w emulsion ointments were formulated to contain 20% petrolatum and/or liquid petrolatum, the water content adjusted to exceed 50%. It was expected that such preparations will be cream-like soft (even pourable) and could be incorporated into a medicated dressing. The procedure used in the preparation of the test ointments was according to the conventional pharmacopeial method as follows.

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[1] The grade official in the "British Pharmacopoeia 1918" which consists of a mixture of diaminoacridine hydrochloride, diaminoacridine dihydrochloride, and hydrochloride of diaminomethyl acridinium chloride; this is not equivalent to the acriflavine official in the "British Pharmaceutical Codex 1934" which, too, differs from acriflavine and acriflavine hydrochloride official in the "National Formulary X" (2). All these derivatives of aminoacridine bear cationic properties.

[2] Aqueous cream (simple cream) B. P. (3) consists of 30% emulsifying ointment B. P. containing anionic sodium lauryl sulfate as the main emulsifying agent.

[3] Inversion point is defined as the temperature at which 1% aqueous solution of the emulsifying agent becomes turbid; inversion temperature of polysorbate 80 is at 85°, whereas that of polyoxyl 30 stearate is at 95°. Mention should be made that with the anionic sodium lauryl sulfate a clear solution is obtained even at 100°.

The oily phase including the emulsifying agent (wherever applicable) was melted on a water bath. The aqueous phase containing acriflavine[4] and the emulsifying agent (wherever applicable) was warmed to the same temperature; both phases were mixed continuously until congealing. The stability controls were conducted stepwise starting with visual observations for any physical changes on storage at room temperature; controls were made at logarithmic intervals starting from 10 seconds and onwards, 100 seconds, 1,000 seconds, 10,000 seconds, 10^5 seconds, 10^6 seconds (11 days, 13 hours, 46 minutes, 40 seconds).[5] Any ointment in which could be detected a perceptible separated oily or aqueous layer was discarded. This was followed by incubation test for 10^6 seconds at 37° (±2°) through which the ointment had to preserve its stability. The last stability test was conducted upon autoclaving (15 lb./inch² pressure, 121°, 10^3 seconds = 16 minutes, 40 seconds). Ointments which failed to be reemulsified to proper consistency, were considered inadequate. The three types of stability controls combined proved valuable in selecting the emulsion bases for acriflavine ointments. Preliminary tests have indicated that the storage tests at 37°, which were advocated by Husa and co-workers (12), will not suffice for the evaluation of a thermostable emulsion. Autoclaving stability tests of emulsions were suggested for nonpharmaceutical emulsions (13) and were employed too in a study of parenteral thermostable fat emulsions (8). The results of our study confirm that thermostability is best ascertained by the autoclaving test.

The emulsion ointments of acriflavine which passed the rigid stability tests were subjected to further physical tests: type of emulsion, melting point, and viscosity.

The o/w type of the emulsion ointments was ascertained by the dye method and water miscibility (14). Melting point was determined by the official B. P. method for fats which was somewhat adapted. Apparent viscosities of the emulsion ointments were determined by three different methods: (*a*) rheological falling sphere method at several temperatures; (*b*) Scott orifice efflux viscometer at the fluid phase above melting temperature with and without pressure; and (*c*) Brookfield Synchro-Lectric rotational viscometer at varying rates of shear and at a wide temperature range.

Sterilization of the Emulsion Ointments.—The petrolatum used in the preparation of "petrolatum gauze" is sterilized at 165–170° for 2 hours according to the official directions of the U. S. P. (18). Oven sterilization at the same temperature for 2.5 hours (15, 17) at least was found reliable for gauze dressings impregnated with petrolatum, oils, and fatty ointments. These sterilization procedures involved a very high temperature for a rather long period which might prove detrimental to the emulsion ointments and their constituents. Emulsion ointments cannot be sterilized by bacterial filtration at their melting temperature since the filtering pores are smaller in diameter than that of most of the internal phase globules. However, the presence of water in the selected emulsion ointment enables steam sterilization (16) at 15 lb./inch² (121°) for 30 minutes; as long as saturated steam is used at its phase boundary (19) this less drastic method is highly safe. Furthermore, this method of sterilization coincided with the rigid autoclaving tests and therefore the selected emulsion ointments proved to be thermosterilizable. The rate of redispersion after autoclaving was not equal for all emulsion ointments but in all cases it has been secured even after repeated sterilizations.

Bacteriostatic Tests.—The antiseptic activity of the acriflavine ointments was evaluated by the bacterial inhibition test (20, 21) which was modified to secure most concordant results. The standard agar cup plate method utilizing *Staphylococcus aureus* was expanded and the ointments were tested similarly with virulent strains of *Escherichia coli*, *Pseudomonos aeruginosa*, and *Streptococcus pyogenes;* since these too appear quite often in skin infections. The ointments were spread in uniform strips of dimensions 50 × 6 mm. on sterile agar plates which were inoculated serially with $^1/_{10}$ and $^1/_{100}$ dilutions of the bacterial culture, and incubated for 24 to 48 hours. In the case of *Streptococcus pyogenes*, controls were made with sterile blood agar plates. Inhibition zones were measured in millimeters from one side of the ointment strip. Acriflavine was incorporated in the selected ointment bases in ascending amounts starting with 0.1% w/w up to 2% w/w; considering the average molecular weight of acriflavine as 281.4, the concentrations were converted into millimole % (m*M* %) and accordingly the range extended between 0.355 m*M* % and 7.10 m*M* %.

Emulsion Bases.—Single and compound formulations with polysorbate 80 did not lead to stable ointment bases. The incorporation of cetostearyl alcohol did not induce stability at 37°. The results with polysorbate 80 did not coincide with that indicated by Benerito-Singleton tests: in spite of the high inversion point of the water solubility of polysorbate 80, autoclavable emulsion ointments could not be prepared with it.

The formulations with Cremophor A fest (solid) combined with Cremophor A flussig (liquid) were not stable at 37°. On the other hand Cremophor A solid as a single emulsifying agent produced an emulsion base stable at 37° but failed at steam sterilization temperature. Cremophor Ap solid combined with Cremophor A solid as recommended by the manufacturer (11) led to a base unstable at 37°.

Preliminary formulations with polyoxyethylene 30 stearate as a single o/w emulsifying agent (even up to 10%) were unsuccessful. Omitting the petrolatum and/or liquid petrolatum and upon incorporation of cetostearyl alcohol (10–25%) and propylene glycol (10–12%) smooth ointments were obtained which, however, were too dry. On further trials, following the U. S. P. XV formula for hydrophilic ointment, we have found that with cetostearyl alcohol 3% w/w, and polyoxyethylene 30 stearate 3% w/w, a suitable thermosterilizable emulsion ointment could be produced (base I). The substitution of cetostearyl alcohol with Cremophor A solid in this base led to a higher viscosity. Screening the many bases of this kind prepared, it was inferred that the most appropriate emulsion base consisted of a blend of polyoxyethylene 30 stearate 5% w/w and Cremophor A solid 5% w/w (base II). Further experimentation indicated that far more stability could be

[4] B. P. 48 grade was used throughout this study.
[5] The theoretical and practical aspects of logarithmic periodical emulsion controls is to be reported in a further communication apart from this study.

achieved (redispersion after autoclaving unnecessary) by adding cetostearyl alcohol to the constituents of base II. The best formulation included cetostearyl alcohol 2.5% w/w, polyoxyethylene 30 stearate 2.5% w/w, and Cremophor A solid 4% w/w (base III).

The reduction of the water content to about 50% without increasing the concentration of the oleaginous phase can be effected by incorporating water-soluble or water-miscible constituents. Polyethylene glycols of molecular weights 400 and 4000, respectively, proved valuable in official ointment formulations but alone are considered to produce improper ointments (6). The emulsification of aliphatic oleaginous ingredients in the presence of polyethylene glycols proceeds rather with some difficulty, and even then the best emulsifying agent was found to be polyoxyethylene 30 stearate. Upon autoclaving some of polyethylene glycol 4000 crystallized out and this had to be replaced by various other polyethylene glycols. Good results were obtained by decreasing the proportion of polyethylene glycol 400 and incorporating polyethylene glycol 900 and 1500, respectively (base IV).

The results with polyoxyethylene 30 stearate confirmed the Benerito-Singleton test for the selection of the main emulsifying agent producing thermostable emulsions.

The consistency of the lecithin o/w emulsion bases were appropriate but proved to be unstable, particularly upon autoclaving; this could have been predicted from the Benerito-Singleton test.

The composition and the physical characteristics, including the stability data of the four selected bases, are listed in Table I. It should be noted that the o/w emulsion type offers washability and that the decline in apparent viscosities of these bases at body temperature enables proper diffusion, spreading, and penetration. The rheograms of these bases at 20° reproduced in Figs. 1 and 2 indicate plastic viscosities with thixotropic features—particularly of bases I and II; owing to the different range of viscosities two separate spindles were used: Spindle II (Brookfield Synchro-Lectric viscometer model HBT) for bases I and II and spindle III for bases III and IV.[6] The rheogram of glycerin (99% w/w) which possesses Newtonian viscosity was also included in Figs. 1 and 2 for comparison.

Antibacterial Effectiveness.—In spite of the oleaginous phase in the selected o/w emulsion bases the antibacterial activity of the incorporated acriflavine was not hindered as compared with that of a greaseless starch glycerite base (see Table II). It was reascertained that the optimal antiseptic concentration of acriflavine in effective ointments bases is about 1% w/w (3.55 m*M* % w/w). Equivalent concentrations of Flavazole (a proprietary equimolecular combination of 2,8-diaminoacridine and sulfathiazole) in one of the selected ointment bases did not possess superior antiseptic activity. Furthermore, the incorporation of 1% sulfathiazole into 1% acriflavine o/w emulsion ointment did not enhance the antibacterial activity. On the other hand, cream of proflavine B. P. C. 1954 (22) which is a w/o emulsion containing only 0.1% proflavine did not inhibit the growth of any of the test organisms.

[6] The thixotropy of bases III and IV is less pronounced and appears only at the lowest r. p. m. (see Fig. 2, base of slope C).

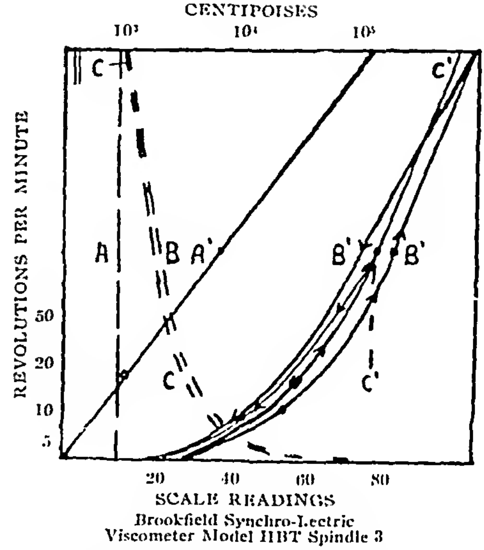

Fig. 1.—Rheogram of bases I and II: Shearing stress (scale readings) and apparent viscosity (centipoises—log scale) *versus* rate of shear (revolutions per minute). ⟷ Slope of shearing stress upon increasing or decreasing rate of shear.- - - - - - - Slope of apparent viscosity upon increasing rate of shear. A, A′, glycerin 99% w/w. B, B′, base I. C, C′, base II.

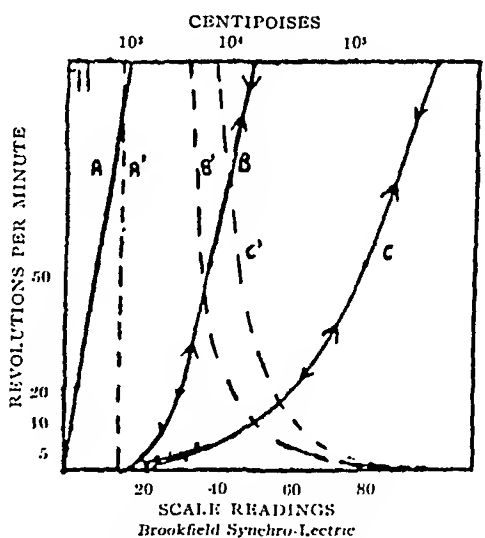

Fig. 2.—Rheogram of bases III and IV: shearing stress (scale readings) and apparent viscosity (centipoises—log scale) *versus* rate of shear (revolutions per minute). ⟷ Slope of shearing stress upon increasing or decreasing rate of shear.- - - - - - Slope of apparent viscosity upon increasing rate of shear. A, A′, glycerin 99% w/w. B, B′, base III. C, C′, base IV.

Preparation of Acriflavine Antiseptic Dressings.—Absorbent gauze is cut in strips and folded according to the pharmacopeial directions for petrolatum gauze (17) and filled into heat-sterilizable wide-mouth

TABLE I.—PHYSICAL CHARACTERISTICS OF ACRIFLAVINE OINTMENTS 1% w/w (3.55 mM %) WITH THE SELECTED EMULSION BASES

Constituents of the Bases	Base I, %	Base II, %	Base III, %	Base IV, %
Nonaqueous phase (oleaginous)	20 w/w	20 w/w	20 w/w	20 w/w
Petrolatum		20 w/w		
Liquid petrolatum	20 w/w		20 w/w	20 w/w
Aqueous phase	74 w/w	70 w/w	71 w/w	74 w/w
Water content	64 w/w	60 v/w	61 v/w	47 v/w
Propylene glycol	10 w/w	10 w/w	10 w/w	10 w/w
Polyethylene glycol 400				3 w/w
Polyethylene glycol 900				4 w/w
Polyethylene glycol 1500				10 w/w
Emulsifying agents	6 w/w	10 w/w	9 w/w	6 w/w
Cetostearyl alcohol	3 w/w		2.5 w/w	3 w/w
Cremophor A solid		5 w/w	4 w/w	
Polyoxyl 30 stearate	3 w/w	5 w/w	2.5 w/w	3 w/w
Consistency	Viscous cream-like (pourable)	Viscous cream-like (pourable)	Soft semisolid (nonpourable)	Very viscous cream-like (pourable)
Melting point	−4°	+1°	44°	−10°
Specific gravity	0.982 (30°)	0.978 (35°)	0.966 (40°)	1.012 (36°)
Type of emulsion	o/w	o/w	o/w	o/w
Stability at 10^6 seconds at room temperature	Stable	Stable	Stable	Stable
Stability at 10^6 seconds at "incubation" 37° (±2°)	Stable	Stable	Stable	Stable
Stability at 10^3 seconds at 121°, 15 lb./inch² (autoclaving)	Stable (redispersion necessary)	Stable (redispersion necessary)	Stable	Stable (redispersion necessary)
Stability at sterilization (121°, 15 lb./inch², 30 min.) as a medicated dressing (gauze impregnated with acriflavine emulsion ointment)	Very slight separation of emulsion phases redispersed by one or two "shakes"	Slight separation of emulsion phases, redispersion effected after intermittent shaking	Most stable preparation (redispersion not necessary)	Separated emulsion phases redispersed after continuous shaking up to congelation
Apparent viscosity decline with increasing temperature	Centipoises	Centipoises[g]	Centipoises	Centipoises
10°	83,200[a]	62,000[b]	208,000[b]	196,800[b]
15°	65,600[a]	58,400[a]	172,000[b]	192,000[b]
20°	51,200[a]	44,800[a]	128,000[b]	188,000[b]
25°	44,000[a]	24,800[a]	112,000[b]	152,000[b]
30°	45,600[a]	22,400[a]	100,000[b]	110,000[b]
30°	30,300[d]			
35°	32,000[a]	20,800[a]	100,000[b]	68,000[b]
35°	..	14,700[d]		
36°				15,100[d]
40°	16,800[c]	4,000[c]	84,000[b]	4,000[c]
40°			9,800[d]	
50°	6.07[e]	6.95[e]	6.61[e]	9.26[e]
50°	2.85[f]	4.22[f]	4.17[f]	4.36[f]

[a] Viscosity measured with Brookfield Synchro Lectric rotational viscometer model HBT, 1 r.p.m., spindle 2.
[b] Viscosity measured with Brookfield Synchro-Lectric rotational viscometer, model HBT, 1 r.p.m., spindle 3.
[c] Viscosity measured with Brookfield Synchro Lectric rotational viscometer, model HBT, 1 r.p.m., spindle 1.
[d] Viscosity measured with falling sphere viscometer (diameter of the sphere 0.3125 inch, weight 2.035 Gm.).
[e] Viscosity measured with Scott orifice efflux viscometer at atmospheric pressure.
[f] Viscosity measured with Scott orifice efflux viscometer at 50 mm. Hg pressure above atmospheric (increased rate of shear).
[g] The apparent viscosities of bases I and II overlap but at most of the rates of shear above 2.5 r.p.m. the apparent viscosities of base II are higher than that of base I (see Fig. 1).
Ung. Glycerin Pharmacopoea Helvetica, 5th ed., as the base of 1% w/w acriflavine ointment is comparatively unstable, glycerin separates out, perceptible mold growth develops.

glass containers. The manner of impregnation of the gauze with the acriflavine emulsion ointment depends on whether a pourable ointment (base I, II, and IV) or a cream-like ointment (base III) is used. In case of the former, the ointment is directly poured to cover the gauze entirely, while with the latter type of ointment it should be premelted (at 44°) before pouring it. The containers are tightly closed and steam sterilized at 121° (15 lb./inch²) for 30 minutes. The applicability of the sterile acriflavine saturated gauze dressing is the same with whichever emulsion ointment it is prepared.

CONCLUSIONS AND SUMMARY

Thermosterilizable oil-in-water emulsion ointments of acriflavine are clinically preferred for topical antiseptic dressings. Emulsion ointments need not be oven-sterilized at 165° for 2 hours since the presence of water enables effective sterilization by autoclaving at 121°, 15 lb/inch², for 30 minutes only. The stability of thermolabile emulsion-ointments is substantially reduced by

TABLE II.—BACTERIAL INHIBITION ZONES[a] OF ACRIFLAVINE OINTMENTS

Bases	Acriflavine Content mM %, w/w	Acriflavine Content Gm %, w/w	*Staph. aureus* 1/10 d[b]	*Staph. aureus* 1/100 d[b]	*E. coli* 1/10 d[b]	*E. coli* 1/100 d[b]	*Ps. aerug.* 1/10 d[b]	*Ps. aerug.* 1/100 d[b]	*Streptococcus pyogenes* 1/10 d[b] A[c]	*Streptococcus pyogenes* 1/10 d[b] B[d]	*Streptococcus pyogenes* 1/100 d[b] A[c]	*Streptococcus pyogenes* 1/100 d[b] B[d]
I	0	0										
	0.35	0.1	2	2					4	4	5	5
	1.78	0.5	5	7				2	5	7	4	8
	3.55	1.0	6	8		2	1	3	4	7	4	8
	5.32	1.5	5	7		3	1	3	7	7	4	8
	7.1	2.0	5	7		3	1	4	6	8	5	8
II	0	0										
	0.35	0.1	3	2					5	4	3	4
	1.78	0.5	4	4					3	4	7	4
	3.55	1.0	4	5				2	5	4	7	5
	5.32	1.5	4	5	1	1	4	4	7	7	6	6
	7.1	2.0	4	5	2	2	5	5	5	7	6	8
III	0	0										
	0.35	0.1	3	3					2	3	5	10
	1.78	0.5	3	7					6	4	5	10
	3.55	1.0	3	7		2	2		6	5	7	10
	5.32	1.5	5	7	2	3	1	3	5	5	7	12
	7.1	2.0	5	7	2	4	1	4	7	6	7	12
IV	0	0										
	0.35	0.1	2	3					4	4	4	3
	1.78	0.5	3	4					7	6	7	8
	3.55	1.0	4	6	1			1	6	6	6	8
	5.32	1.5	5	6	1	1	1	1	5	6	6	8
	7.1	2.0	5	5	1	1	2	2	5	6	6	8
Ung. Glycerin[e]	0	0										
	0.35	0.1	1	2	1	2	1	1	2	3	3	4
	1.78	0.5	3	4	1	3	2	1	4	5	5	6
	3.55	1.0	4	5	2	3	1	1	5	5	5	6
	5.32	1.5	4	5	2	1	1	1	5	6	6	6
	7.1	2.0	5	5	1	2	1	2	6	6	6	7

[a] Inhibition zones measured in millimeters, from one side of the ointment strip. [b] d = dilutions. [c] A = Simple Agar. [d] B = Blood Agar. [e] Ung. Glycerin Pharmacopoea Helvetica: Amyl. Trit. 10 parts, Aq. dest. 15 parts, Glycerin 90 parts.

subjecting them to steam sterilization and the decomposition which ensues is irreversible.

Thermostable sterilizable emulsions could be prepared by utilizing selected emulsifying agents properly blended. High temperature inversion point of an aqueous solution of an emulsifying agent *per se* signifies possible thermostability, while actual autoclave-thermotesting of the whole emulsion system containing the emulsifying agent determines conclusive thermostability. Both polysorbate 80 and polyoxyethylene 30 stearate exhibit inversion points at comparatively high temperatures. However, it was found that only polyoxyethylene 30 stearate mixed with auxiliary dispersion agents could produce thermosterilizable emulsion bases from which four bases of proper consistency were selected for the preparation of sterile acriflavine ointments.

The bacteriostatic activity of the sterile acriflavine emulsion ointments prepared with the selected bases is equivalent to that obtained with greaseless starch glycerite base with equal concentrations of acriflavine. It was confirmed that the least efficient concentration of acriflavine in water-miscible ointments is 1% which equals to 3.55 millimole %.

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A Study of Meprobamate-Curare Interaction*

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Since both meprobamate and curare working through different sites and modes of action can produce the same gross effect of skeletal muscular relaxation, the possibility of synergistic interaction was investigated. The existence of such synergy has been demonstrated as an increased effectiveness and/or toxicity of curare in guinea pigs, rats, and mice pretreated with meprobamate.

Although meprobamate (Miltown, Equanil. 2-methyl-2-*n*-propyl-1,3-propanediol dicarbamate) was synthesized (1, 2) and originally investigated as a muscle relaxant (3–5), that aspect of its pharmacology was soon overshadowed in the public attention by its usefulness as a tranquilizer (6–8) Many clinical and laboratory investigators, however, have maintained interest in the muscle relaxant properties of this drug

Clinical experience has shown beneficial responses in patients with cerebral palsy (9–11) and in patients with skeletal muscle spasm (12) Improvement has been seen in the general muscular coordination of patients and in their ability to perform tasks (13). In studies with laboratory animals, meprobamate has been used to control experimental epilepsy (14), and it has been shown that the muscular relaxation from meprobamate is potentiated by phenobarbital and extract of hyoscyamus (15, 16).

Investigation of the interaction between meprobamate and other drugs is of considerable import, not only because of the widespread use of the drug as a tranquilizer, but also because of the growing tendency to administer two or more drugs of the therapeutic armamentarium concomitantly Such investigations have been underway at our laboratories since 1956 (17).

Study of interaction between meprobamate and curare seemed desirable from a practical viewpoint because curariform drugs are commonly employed in medical practice They are established agents as adjuncts to general anesthesia as well as in convulsive shock procedures in psychiatric therapy Furthermore, numerous European papers have recommended meprobamate as a preanesthetic medication in obstetrics (e g, 18), and it is evident that occasional obstetrical patients become surgical patients, requiring general anesthesia Meprobamate has also been suggested as a preoperative medication (19).

Pharmacologically, meprobamate and curare can produce the same gross effect of skeletal muscle relaxation but do so by completely different mechanisms (20, 21). Tubocurarine is a relatively short acting drug at the myoneural junction while meprobamate is a long acting drug in multineuronal circuits Theoretically, synergy can be expected from two drugs which produce the same gross effect through different sites of action (22) It therefore seemed probable that there would be an interaction between meprobamate and those drugs which interrupt myoneural transmission

MATERIALS AND METHODS

Animals were obtained from different sources and were usually adult males They were fed commercial diets supplemented with fresh vegetables and kept in the thermostatically regulated animal house for several weeks prior to use For each comparison conducted, the control and experimental animals used were from the same supplier, of the same sex, strain, and size range

All drugs[1] were obtained in solution except for meprobamate which was suspended in 5% acacia solution, and were administered by intraperitoneal injection The doses used were within the range of those employed by a great many other workers (e g, 23, 24), the largest dose of meprobamate being 100 mg/Kg Since loss of righting reflex is an important criterion in this study, it must be pointed out that in hundreds of experiments in our laboratories, this dose was completely ineffective in inducing a loss of righting reflex in guinea pigs, rats, and mice Meprobamate was always injected thirty minutes prior to administration of the curariform agent after preliminary experiments showed that simultaneous administration of the slow-acting meprobamate with the rapidly-acting curariform drug was impractical

Experiments were always conducted with paired groups of animals on the same day Times were all taken to the nearest minute The time for onset of paralysis was recorded when the righting reflex of the head was lost, and the time of recovery was noted at the reappearance of this reflex The time of death was recorded at the cessation of heartbeat.

When there were qualitative differences in responses, the significance of difference between proportions was calculated. When qualitative re-

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[1] *d*-Tubocurarine chloride (Tubarine, Burroughs Wellcome), dimethyl tubocurarine chloride (Mecostrin, Squibb), dimethyl tubocurarine iodide (Metubine, Lilly), *Chondodendron tomentosum* extract (Intocostrin, Squibb), and meprobamate (Miltown, Wallace) were supplied through the courtesy of their manufacturers

TABLE I.—MEPROBAMATE AND CURARE IN MALE GUINEA PIGS

Treatment, mg./Kg. Except Intocostrin, Units/Kg.	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death	Type Synergy and Significance
2.0 Intocostrin	10	10	0	0	Increased effect
100 Miltown + 2 Intocostrin	10	5	4	1	$P < 0.02$
0.05 Mecostrin	10	10	0	0	Increased effect
100 Miltown + 0.05 Mecostrin	10	0	9	1	$P < 0.001$
0.1 Metubine	20	2	7	11	Increased toxicity
100 Miltown + 0.1 Metubine	20	1	1	18	$P < 0.05$
0.4 Tubarine	10	0	5	5	Increased toxicity
100 Miltown + 0.4 Tubarine	10	0	0	10	$P < 0.02$

The number of animals affected was significantly increased when meprobamate was combined with Intocostrin or Mecostrin and the number of deaths was significantly increased when meprobamate was combined with the other two drugs

TABLE II.—MEPROBAMATE AND CURARE IN MALE MICE

Treatment mg./Kg. Except Intocostrin, Units/Kg.	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death	Type Synergy and Significance
4.0 Intocostrin	10	7	0	3	Increased toxicity
100 Miltown + 4 Intocostrin	10	0	1	9	$P < 0.02$
0.4 Mecostrin	10	9	1	0	Increased toxicity
100 Miltown + 0.4 Mecostrin	10	0	3	7	$P < 0.01$
0.6 Metubine	10	5	1	4	Increased effect
100 Miltown + 0.6 Metubine	10	0	4	6	$P < 0.02$
0.4 Tubarine	10	10	0	0	Increased toxicity
100 Miltown + 0.4 Tubarine	10	4	0	6	$P < 0.01$

The number of animals affected was significantly increased when meprobamate was combined with Metubine, and the number of deaths was significantly increased when meprobamate was combined with the other three drugs

TABLE III.—MEPROBAMATE AND CURARE IN MALE RATS

Treatment mg./Kg. Except Intocostrin Units/Kg.	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death	Type Synergy and Significance
4.0 Intocostrin	7	0	0	7	Decreased onset
100 Miltown + 4 Intocostrin	7	0	0	7	time $P < 0.05$
0.1 Mecostrin	10	0	0	10	Decreased survival
100 Miltown + 0.1 Mecostrin	10	0	0	10	time $P < 0.001$
0.01 Metubine	10	10	0	0	Increased effect
100 Miltown + 0.01 Metubine	10	5	4	1	$P < 0.02$
0.2 Tubarine	11	0	8	3	Increased toxicity
100 Miltown + 0.2 Tubarine	11	0	3	8	$P < 0.05$

The number of animals affected was significantly increased when meprobamate was combined with Metubine and the number of deaths was significantly increased when meprobamate was combined with Tubarine The time of onset of action of 5.1 $\pm$0.5 minutes for Intocostrin was significantly reduced to 3.1 + 0.1 minutes by pretreatment with meprobamate The length of time of survival of 10.1 $\pm$ 0.1 minutes for Mecostrin was reduced to 6.1 $\pm$ 0.7 minutes by pretreatment with meprobamate

TABLE IV.—ACUTE TOXICITY TUBARINE ALONE AND FOLLOWING PRETREATMENT WITH MILTOWN (GROUPS 10 GUINEA PIGS)

Tubarine: mg./Kg.	Tubarine: Mortality	Tubarine: LD$_{50}$, mg./Kg.	100 mg./Kg. Miltown + Tubarine: mg./Kg.	100 mg./Kg. Miltown + Tubarine: Mortality	100 mg./Kg. Miltown + Tubarine: LD$_{50}$, mg./Kg.
0.30	0		0.10	1	.
0.35	2		0.20	4	
0.40	5	0.42	0.30	6	0.24
0.45	6		0.40	10	
0.50	7				

The difference between the means of the doses lethal for animals pretreated with meprobamate and those not so pretreated is very highly significant with $P < 0.001$ This may be interpreted to mean that pretreatment with 100 mg./Kg meprobamate thirty minutes before administration of Tubarine was equivalent to a 75% increase in the dose of Tubarine

sponses were the same for all animals of both groups (e. g., all were paralyzed and recovered, or all died). *t* tests were applied to demonstrate the significance of quantitative differences The times for onset of

TABLE V.—CROSSOVER TREATMENT IN FEMALE RATS

Group	Treatment, mg./Kg.	No. of Animals	Effect: Paralysis and Recovery	Effect: Paralysis and Death
A	0.2 Tubarine	8	7	1
B	100 Miltown + 0.2 Tubarine	8	3	5
A Survivors	100 Miltown + 0.2 Tubarine	7	0	7
B Survivors	0.2 Tubarine	3	3	0

Following the initial treatments the difference in percentage fatalities was not significant, but there was a significant difference in times of onset of paralysis for the two groups, with $P < 0.02$ When the crossover was done three days later, all animals which had survived tubocurarine alone were killed by the combination, and no animal which had survived the combination was killed by the tubocurarine alone, $P < 0.01$.

TABLE VI.—EFFECTS OF SMALL DOSES ON GUINEA PIGS AND RATS

Treatment, mg./Kg.	Species	No. of Animals	Effect: No Paralysis	Effect: Paralysis and Recovery	Effect: Paralysis and Death
25 Miltown + 0.2 Tubarine	G. P.	7	7	0	0
50 Miltown + 0.2 Tubarine	G. P.	7	6	1	0
75 Miltown + 0.2 Tubarine	G. P.	7	3	4	0
0.1 Tubarine	Rat	8	8	0	0
50 Miltown + 0.1 Tubarine	Rat	8	6	2	0
100 Miltown + 0.1 Tubarine	Rat	8	5	2	1
0.2 Tubarine	Rat	8	3	3	2
50 Miltown + 0.2 Tubarine	Rat	8	0	4	4

With guinea pigs, results from pretreatment with 50 mg./Kg were not significantly greater than with 25 mg./Kg., nor were results with 75 mg./Kg. significantly greater than with 50 mg./Kg., but pretreatment with 75 mg./Kg. was significantly more effective than with 25 mg./Kg., $P < 0.05$. Although these combinations were apparently nontoxic, none was consistently effective, and since 100 mg./Kg. meprobamate plus 0.2 mg /Kg., Tubarine had previously killed 40% of the test animals, it did not appear worthwhile to pursue the search for a combination of dosages that would be both consistently effective and uniformly safe in guinea pigs. With rats, there was likewise no substantial margin of safety; no combination which could be relied upon to produce paralysis without producing deaths.

paralysis, duration of paralysis, and survival are recorded as the mean time plus or minus the standard error.

RESULTS AND DISCUSSION

Synergy between meprobamate and curare in guinea pigs, mice, and rats can be seen from the results given in Tables I, II, and III.

In addition to the synergistic effects previously recorded, a change in the LD_{50} can be demonstrated by expanding the range of doses, as illustrated by Table IV.

A comparison of the results in the previous table shows that a nontoxic dose of meprobamate (100 mg./Kg.) followed by a nontoxic dose of tubocurarine (0.1, 0.2, 0.3 mg./Kg.) formed a highly dangerous combination for these guinea pigs. When crossover tests were conducted to compare the actions of curare alone and curare after meprobamate in the same animals, the results were always the same; the combination of drugs was more effective, i. e., more toxic. This type of response is illustrated in Table V.

In the hope that a reliably effective, nontoxic combination of doses might be obtained, smaller doses of meprobamate were used as pretreatments, and pretreatments were given before smaller doses of curare. This type of experiment is illustrated by Table VI.

It was, however, possible to observe a subparalytic interaction of the drugs. That is, a dose of tubocurarine which had no subjectively determinable effect on rats could be seen to produce muscular relaxation in rats which had been pretreated with a small dose of meprobamate. When rats were given 0.15 mg./Kg. tubocurarine, there was no obvious gross effect on the musculature or activity. At the same time, rats pretreated with 20 mg./Kg. meprobamate were given 0.15 mg./Kg. tubocurarine and although none of this group became paralyzed, their musculature and activity were both affected. These latter animals flattened down in the bottom of the cage and could be pushed about readily by hand, although they resisted being placed on their sides. The amount of meprobamate used in this experiment does not in itself have a grossly demonstrable effect on the skeletal muscle of the rat.

Apparently this synergistic type of action with meprobamate is not limited to the curares, but extends to the curarimimetic drugs. Thus, we have seen significant interaction with succinylcholine chloride and with gallamine trimethiodide, and presume that it is a general effect.

SUMMARY

The studies described have demonstrated the synergy between meprobamate and curare. The toxicity from a given dose of curare was increased in animals which had been pretreated half an hour before with a nontoxic, nonparalytic dose of meprobamate.

During these studies, no combination of dosages of these drugs was encountered which would paralyze all animals without killing any.

It was possible, however, to observe a nonparalytic decrease in skeletal muscle tone of the rat after a combination of a low, ineffectual dose of meprobamate followed by a low, ineffectual dose of tubocurarine.

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Isotonic Solutions VIII*

The Permeability of Red Corpuscles to Various Salts of Gluconic Acid

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A comparison was made of van't Hoff *i* values calculated from hemolytic and freezing point data for various salts of gluconic acid. Sodium, potassium, and manganese(II) gluconates gave higher *i* values by the hemolytic method than by the freezing point depression method; iron(II) and cobalt(II) gluconates gave lower *i* values by the hemolytic method. Magnesium and calcium gluconates gave higher *i* values by the hemolytic method with human erythrocytes but not with rabbit erythrocytes. Zinc gluconate gave extremely high hemolytic *i* values due to the partial precipitation of the oxyhemoglobin liberated from laked erythrocytes. Hemolytic *i* values of the gluconates were generally lowered when determined in the presence of sodium chloride. Erythrocytes from Negro donors were, on the average, more resistant to osmotic hemolysis than those from Caucasian donors. Results substantiate the premise that solutions calculated to be isosmotic with blood according to colligative property data are not necessarily isotonic to the red corpuscle.

The adjustment of solutions intended for intravenous administration to the same tonicity as blood is of utmost importance. The advantages of employing the hemolytic method in the preparation of such solutions have been emphasized by Husa and co-workers (1–7). They pointed out that the osmotic effect of a substance on the erythrocyte depends not only on the concentration of the substance but also on whether or not it penetrates or affects the properties of the cell membrane. Thus, solutions calculated to be isosmotic with blood according to physicochemical data are not necessarily isotonic to the red blood cell.

Cadwallader and Husa (8) recently reported that zinc acetate gave unusually high hemolytic *i* values, much beyond that calculated from colligative property data. Their work complemented that of Hartman and Husa (4), who found similarly high hemolytic *i* values for zinc sulfate.

The primary purpose of the present work was to investigate the elements closely related to zinc in the periodic table to determine whether or not their action paralleled that of zinc. Added interest in the investigation was stimulated by the fact that these elements, i. e., copper, cobalt, iron, and manganese, along with zinc are so-called "trace elements," essential to human nutrition. To obtain a comparative picture of the activities of these elements, they were all employed as salts of gluconic acid. Sodium, potassium, magnesium, and calcium gluconates were also studied and their hemolytic *i* values determined. The *i* values obtained by the hemolytic method were compared to *i* values calculated from freezing point depression data collected in this laboratory.

EXPERIMENTAL

Collection of Blood.—The rabbit and human bloods employed in this investigation were obtained in the manner described by Grosicki and Husa (2). The human blood was drawn chiefly from the veins of the arms of a twenty-five-year-old male, although some experiments were conducted employing blood donated by male volunteers.

Preparation of Solutions.—Solutions of the chemicals were prepared by placing the proper amounts in volumetric flasks which were then brought to volume with triple-distilled water. An approximate concentration range was determined for each gluconate by conducting a preliminary experiment in which the concentration of gluconate just sufficient to prevent hemolysis and the maximum concentration present without the prevention of hemolysis constituted the limits of the desired range. Various intermediate concentrations were then prepared and the experiment carried out. All of the chemical concentrations were calculated in terms of the anhydrous salt.

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis was that of Hunter (9), as adapted for the determination of *i* values of salts forming colorless solutions by Grosicki and Husa (2). Several gluconates studied in the present investigation formed colored aqueous solutions, e. g., cobalt(II), copper(II), iron(II), and manganese(II) gluconates. For the quantitative determination of the per cent hemolysis when studying such solutions, one of two methods was employed depending on whether or not the color of the gluconate solution produced an additive colorimetric reading with oxyhemoglobin solution.

In the first method, oxyhemoglobin solution pre-

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The gluconates used in this study were generously supplied by Chas. Pfizer & Co., Inc., Brooklyn 6, N. Y., and Chemo Puro Manufacturing Corp., Newark 5, N. J.

pared by laking erythrocytes in triple distilled water was added in like amounts to each of two colorimeter tubes, one containing 5 ml of triple distilled water and the other containing 5 ml of the gluconate solution. To a third tube, containing 5 ml of the gluconate solution, a portion of triple distilled water equal in volume to the oxyhemoglobin solution placed in each of the first two tubes was added. The colorimetric reading of the third tube added to the reading of the tube containing only water and oxyhemoglobin was compared to the reading of the tube containing the oxyhemoglobin and the colored gluconate solution; if the readings agreed within experimental error, the two colors were considered to be additive. If this were found to be the case, colorimeter readings of the gluconate solutions were taken before the addition of blood and served as blank readings. These blank readings subtracted from the colorimetric readings obtained at the end of the hemolytic experiment permitted an accurate determination of the proportion of light absorbed by the oxyhemoglobin present in the supernatant liquid.

If the colorimetric readings of the oxyhemoglobin and the gluconate solutions were not found to be additive, the second of the two methods devised was employed. In this method the per cent hemolysis was determined by difference, that is, from the proportion of oxyhemoglobin remaining in the unlaked erythrocytes. The standard procedure used when employing colorless solutions was carried out until just prior to the time that the colorimetric readings were to be taken. Instead of taking the readings, the supernatant solution, containing the colored gluconate and oxyhemoglobin from laked cells, was meticulously decanted from each tube, being careful not to disturb the unhemolyzed cells spun to the bottom of the tubes. The unhemolyzed cells were then washed with 0.85% sodium chloride solution, recentrifuged, and the supernatant liquid decanted, the process being repeated until the washings were free of color. The supernatant liquid of the last washing was replaced with 0.1% sodium carbonate solution added to a mark etched on the colorimeter tube, denoting a volume of 5.05 ml. The tube was inverted several times to insure complete hemolysis, the ghost cells centrifuged down, and the resulting color read with the colorimeter. By subtracting the average reading of two duplicate tubes from the average reading of 100% hemolysis, the colorimetric value of the oxyhemoglobin exuded from the cells originally hemolyzed in the gluconate solution was obtained. From this value the per cent of hemolysis was calculated in the usual manner (2). All of the hemolysis experiments were conducted at 37° ± 1°.

Calculation of *i* Values from Per Cent Hemolysis Data.—The calculation of hemolytic *i* values has been explained by Husa and co-workers (2, 3).

Determination of Metal-Protein Precipitation.—To aid in the interpretation of certain hemolytic *i* values, tests were carried out to determine whether or not precipitation occurred when solutions of oxyhemoglobin were added to solutions of the gluconates. One % solutions of each gluconate were separately mixed with equal portions of oxyhemoglobin solution prepared by laking washed erythrocytes, allowed to stand in a water bath at 37° for forty-five minutes, centrifuged, and observed for evidence of precipitation. During the course of a hemolytic run, such precipitation would cause the colorimetric readings to be proportionally lower than those which would normally result from the proportion of oxyhemoglobin actually released from the laked erythrocytes.

Additional experiments were carried out to determine whether or not serum proteins were precipitated by gluconate solutions. Serum was separated from blood cells by centrifugation and added in like portions to 1% solutions of each gluconate. The mixtures were observed repeatedly for forty-five minutes during which time they were in a water bath at 37°.

Freezing Point Determinations.—The apparatus used to determine the freezing points of 0.010 molal (for calcium gluconate) and 0.025 molal gluconate solutions was that of Bartley (10), as modified by Husa and Adams (1). The average freezing point depression of water for ten samples of each gluconate solution was determined and the van't Hoff *i* values calculated according to the following formula

$$\Delta T_f = K_f i M$$

where ΔT_f is the change in the freezing point of water after the addition of solute, K_f is the molal freezing point constant of water (1.86), i is the van't Hoff factor, and M is the molality of the solution.

RESULTS AND DISCUSSION

Results With Sodium and Potassium Gluconates.—Employing the hemolytic method, sodium and potassium gluconates gave higher *i* values than would be expected for compounds yielding two ions. Freezing point information indicated that each of these gluconates acted normally, i. e., as slightly less than two particles, see Table III.

Sodium gluconate exhibited an average *i* value of 2.27 using rabbit blood and 2.37 using human blood. These values were lowered to 1.99 with rabbit blood and 2.07 with human blood when determined in the presence of 0.2% sodium chloride.

Potassium gluconate gave an average *i* value of 2.26 using rabbit blood and 2.28 using human blood. These values were lowered to 2.05 with rabbit blood and 2.13 with human blood when the determinations were conducted in the presence of 0.2% sodium chloride, see Tables I and II.

In solutions of some substances, erythrocytes may lose electrolyte from within the cell, thus causing a decrease in the internal osmotic pressure and a corresponding increase in resistance to hemolysis (2, 11, 12). The exosmosis of erythrocytes in such solutions can be corrected in some cases by the addition of small proportions of an electrolyte, e. g., sodium chloride (2, 12).

The high hemolytic *i* values obtained for sodium and potassium gluconates may be attributed to exosmosis of electrolyte from within the erythrocytes. The lowering of the *i* values upon the addition of sodium chloride may be due to the partial restoration of the environment normal to the red blood cells.

Results With Calcium and Magnesium Gluconates.—The hemolytic *i* values for calcium gluconate, calculated from concentrations causing 25, 50, and 75% hemolysis of human erythrocytes, increased by approximately 0.2 (2.54 to 2.72) going from 25 to 50% hemolysis and another 0.2 (2.72

TABLE I.—VALUES OF i FOR VARIOUS GLUCONATES CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

Gluconate	Hemolysis, % 25	50	75	Averages
Sodium	2.18	2.26	2.36	2.27
Sodium[b]	1.96	1.97	2.05	1.99
Potassium	2.19	2.24	2.34	2.26
Potassium[b]	2.00	2.07	2.08	2.05
Magnesium	2.48	2.64	2.76	2.63
Magnesium[b]	3.03	3.19	3.30	3.17
Calcium	2.32	2.40	2.50	2.41
Calcium[b, c]	2.89	2.96	3.12	2.99
Manganese (II)	2.48	2.54	2.62	2.55
Manganese (II)[b]	2.04	2.18	2.36	2.19
Iron(II)	1.22	1.28	1.32	1.27
Iron(II)[b]	0.93	1.11	1.69	1.24
Cobalt(II)	2.21	2.27	2.48	2.32
Zinc	793.00	933.00	1027.00	918.00

[a] Unless otherwise indicated all i values represent an average of two blood samples
[b] Values determined in the presence of 0.2% sodium chloride
[c] Average of three blood samples

TABLE II.—VALUES OF i FOR VARIOUS GLUCONATES, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

Gluconate	Hemolysis, % 25	50	75	Averages
Sodium	2.32	2.36	2.42	2.37
Sodium[b]	2.01	2.07	2.14	2.07
Potassium	2.20	2.28	2.36	2.28
Potassium[b]	2.08	2.13	2.18	2.13
Magnesium[c]	3.36	3.94	4.69	4.00
Magnesium[b]	3.34	3.40	3.56	3.43
Calcium	2.54	2.72	2.95	2.74
Calcium[b]	2.41	2.56	2.89	2.62
Manganese(II)	2.64	2.70	2.76	2.70
Manganese(II)[b]	2.28	2.37	2.47	2.37
Iron(II)	1.45	1.58	1.79	1.61
Iron(II)[b]	0.74	1.12	2.56	1.47
Cobalt(II)	1.98	2.08	2.18	2.08
Zinc	..[d]	499.00	666.00	582.00

[a] Unless otherwise indicated all i values represent an average of two blood samples
[b] Values determined in the presence of 0.2% sodium chloride.
[c] Average of three blood samples
[d] Data unobtainable at 25% hemolysis

to 2.95) going from 50 to 75% hemolysis. A similar observation was made by previous investigators of calcium gluconate, acetate, and levulinate (5). This steady increase did not occur when the i values for calcium gluconate were determined in the presence of 0.2% sodium chloride, see Table II.

Hemolytic i values for calcium gluconate obtained with rabbit blood increased by approximately 0.1 (2.32 to 2.40) going from 25 to 50% hemolysis and by another 0.1 (2.40 to 2.50) going from 50 to 75% hemolysis. Increases of similar magnitude occurred when the determinations were conducted in the presence of 0.2% sodium chloride.

The presence of 0.2% sodium chloride caused an increase in the i values for calcium gluconate using rabbit blood and a decrease in the i values when human blood was employed. In this respect, magnesium gluconate acted similarly to calcium gluconate.

TABLE III.—VALUES OF i FOR VARIOUS GLUCONATES, CALCULATED FROM FREEZING POINT DATA OBTAINED WITH A MODIFIED BARTLEY APPARATUS[a]

Gluconate	Average Freezing Point Depression, °C.	i Value
Sodium	0.087	1.87
Potassium	0.085	1.83
Magnesium	0.130	2.80
Calcium[b]	0.050	2.69
Manganese(II)	0.102	2.19
Iron(II)	0.114	2.45
Cobalt(II)	0.112	2.41
Copper(II)	0.069	1.48
Zinc	0.119	2.57

[a] Unless otherwise indicated data were obtained from 0.025 molal gluconate solutions
[b] Data obtained from 0.010 molal solutions

The i values obtained for magnesium gluconate were higher in every analogous case than those obtained for calcium gluconate. Employing human blood, an average i value of 4.00 was obtained for magnesium gluconate which was reduced to 3.43 when determined in the presence of 0.2% sodium chloride. These i values are much beyond that calculated for magnesium gluconate from freezing point data, *viz* 2.80. Using rabbit blood, the i values for magnesium gluconate increased from 2.63 to 3.17 when the determinations were carried out in the presence of 0.2% sodium chloride

Under normal conditions, calcium does not penetrate the red blood cell (13) According to Ponder (14) there is considerable exchange of internal potassium for alkaline earth metals in human cells under certain conditions. Davson and Danielli (15) noted the difference in the response of the cat erythrocyte membrane to sodium and potassium permeability in the presence of the alkaline earths; they showed that tonicity changes were responsible for only a portion of the abnormal reactions of the membrane The solidifying influence of calcium has often been noted (16); the alkaline earths were said to thicken the erythrocyte membrane by forming insoluble salts with the phosphatide acids of the cell wall (17) Danielli (18) mentioned that there is a good deal of evidence that substances such as magnesium act primarily on cell surfaces, in some way modifying the excitability of the cells

It is not unlikely that the results obtained for magnesium and calcium gluconates were largely due to the action of the cations on the cell membrane, in some manner altering the normal permeability characteristics of the cell. The presence of 0.2% sodium chloride along with the alkaline earth gluconates seemed to promote exosmosis of rabbit erythrocytes and to prevent it with human erythrocytes

Results With Cobalt(II) and Manganese(II) Gluconates. —Cobalt(II) gluconate gave i values of 2.32 using rabbit erythrocytes and 2.08 with human erythrocytes; freezing point data indicated an i value of 2.41. The lower i values obtained by the hemolytic method may be attributed, in part, to the acidity of the cobalt(II) gluconate solutions (about pH 5); such acidity could cause hemolysis independent of the osmotic pressure of the solution. In solutions containing 0.6% sodium chloride and 2% cobalt(II) gluconate, there was approximately 10% hemolysis of human erythrocytes and essentially no

hemolysis of rabbit erythrocytes, the hemolysis of the human erythrocytes would be necessarily due to something other than osmotic effects and could account for the lower hemolytic i values obtained with human blood than with rabbit blood.

Manganese(II) gluconate gave i values of 2.55 with rabbit erythrocytes and 2.70 with human erythrocytes. In the presence of 0.2% sodium chloride, the i values obtained using each blood type were lowered by approximately the same amount, i. e., 0.33 with human blood and 0.36 using rabbit blood. The lower i values more closely resembled the i value obtained for manganese(II) gluconate by the freezing point depression method, *viz.*, 2.19.

Results With Iron(II) Gluconate.—Employing the hemolytic method, iron(II) gluconate gave i values of 1.27 with rabbit blood and 1.61 with human blood. These i values were lowered to 1.24 using rabbit blood and 1.47 with human blood when the determinations were carried out in the presence of 0.2% sodium chloride. These i values are all lower than would be expected for a compound yielding three ions, an i value of 2.45 was calculated for iron(II) gluconate from freezing point data.

Throughout the course of this investigation, there was a tendency to be doubtful of the stability of the iron(II) gluconate solutions. Stone (19) stated that although iron(II) gluconate is stable in the solid state, its solutions are rather unstable. They are prone to oxidation, with iron(II) changing to iron (III), the rate of transformation being dependent on the pH, temperature, degree of aeration, presence of oxidizing or reducing agents, etc. Oxidation causes a darkening of the solutions, a characteristic which would make certain colorimetric assays unreliable. An iron(III) compound of unknown composition has been found to precipitate from aqueous solutions of iron(II) gluconate as a result of a photochemical reaction.

Although iron(II) gluconate solutions were prepared just prior to use, precipitates were occasionally noticed in the colorimeter tubes during some experiments. These brown colored precipitates generally occurred in the iron(II) gluconate blood mixtures containing the lower proportions of the iron(II) salt. If a portion of the iron(II) gluconate were precipitated as an oxidized form, fewer particles would be in solution to aid in the prevention of osmotic hemolysis, the resulting data would indicate less protection and thus a low i value.

The binding of iron(II) gluconate by plasma proteins (e. g., beta globulins), with or without precipitation, would decrease the number of particles in solution and the resulting i values would be lower than expected. The pH of the iron(II) gluconate solutions was approximately 4.5, remaining about the same during the course of each determination, this acidity could account, to a small degree, for the low i values. In the presence of 0.6% sodium chloride, iron(II) gluconate seemed to cause appreciable hemolysis of human erythrocytes but not of rabbit erythrocytes, in the experiments employing human blood, however, precipitation was observed.

Results With Copper(II) Gluconate.—Copper(II) gluconate in concentrations of from 1 to 10% seemed to cause denaturation of blood, oxyhemoglobin released from hlked erythrocytes turned brown as did the unhemolyzed red blood cells. The pH of the copper(II) gluconate solutions was approximately 4.0, this pH remaining fairly constant for the duration of the experiments. Bloch and Oelsner (20) reported that at pH 3.0 hemoglobin is instantaneously converted to brown hematin, at pH 3.8 it is converted within twenty five minutes, and at pH 4.6 the formation of hematin is incomplete within twenty-four hours. They stated that the rate of conversion is directly proportional to the hydrogen ion concentration.

In the tests conducted to determine the protein precipitating action of copper(II) gluconate, it was found that both rabbit and human blood serum protein were precipitated to a certain extent by this gluconate. The copper(II) gluconate serum protein precipitate redissolved on standing (and instantaneously on agitation) and was milk-white in color. The addition of alkali to the copper(II) gluconate serum mixture resulted in a voluminous precipitate. Ressler, *et al.* (21), found that copper combined in definite proportions with the albumin and gamma globulin of blood serum.

Although hemolytic i values were unobtainable due to the darkening of the blood, freezing point data gave an i value of 1.48 for copper(II) gluconate which was appreciably lower than the usual value of 2.70 for a compound yielding three ions.

Results With Zinc Gluconate.—Employing the hemolytic method, extremely high i values were obtained for zinc gluconate. The values of i at concentrations causing 25, 50, and 75% hemolysis of rabbit erythrocytes were 793, 933, and 1,027, respectively, whereas concentrations causing 50 and 75% hemolysis of human erythrocytes showed i values of 499 and 666, respectively. Values of i at 25% hemolysis of human erythrocytes were unobtainable.

Throughout the course of this investigation, unusual behavior was noticed between blood and zinc gluconate solutions. Blood appeared to agglutinate as it was added to colorimeter tubes containing solutions of the zinc salt, at the same time, a milk white formation presented itself around the blood. Before inverting the tubes (to mix their contents) a layer of clear oxyhemoglobin solution was observed at the bottom of some of the tubes, after inverting the tubes, the contents became uniformly opaque. A sediment was observed at the bottom of each tube after centrifugation which was of a volume greater than could be attributed to the small proportion of blood added. The substance was of a reddish color (resembling cosmetic rouge) and was amorphous under the microscope. The degree of hemolysis was determined in the usual manner and the i values calculated according to the general methods.

Oxyhemoglobin solution was partially precipitated when placed in contact with a 1% solution of zinc gluconate. The precipitate was soluble below pH 5.5 and above pH 10.0, it appeared to be least soluble at about the neutral point. Oxyhemoglobin solutions prepared from human erythrocytes acted in a similar manner. Human and rabbit serums were each precipitated in the presence of zinc gluconate. The white, curdy precipitates were soluble below pH 5.0. Oxyhemoglobin was more effectively precipitated by zinc gluconate when a proportion of serum was present. Also, when serum and oxyhemoglobin were separately added to a zinc gluco-

nate solution, the precipitation was less complete than when the two proteins were mixed before being added. Rawlinson (22) described a zinc oxyhemoglobin compound precipitated from a solution of rabbit blood pigment by zinc sulfate. Gurd (23) said that both gamma globulin and beta lipoprotein can be precipitated in the presence of zinc acetate, with the former aiding in the precipitation of the latter.

When blood was added to a solution containing 1% of zinc gluconate and 0.85% of sodium chloride, the blood cells settled to the bottom of the tube and failed to hemolyze after forty-five minutes at 37°. After centrifugation, the cells were bound together at the bottom of the tube and failed to hemolyze after the supernatant solution was decanted and replaced with triple distilled water. Inversion of the tubes caused the cells to pass through the water, still bound together, and resembling a "pancake." Gibson (13) reported that red cells take up zinc very readily; whether the zinc enters the cells or is bound to the membrane is not known. It has been shown, however, that the zinc cannot be removed by washing in isotonic sodium chloride solution.

It appears obvious that the high hemolytic i values obtained for zinc gluconate in this work and for zinc sulfate and acetate in other works (4, 8) have been due to the precipitation of the oxyhemoglobin liberated from laked erythrocytes which made the colorimetric readings of the degree of hemolysis misleading.

Variability of Blood.—The osmotic fragility of erythrocytes varied from animal to animal and, indeed, from day to day in the same animal, employing blood from the same donor, diurnal variations were observed in the degree of hemolysis occurring in a given concentration of sodium chloride solution.

Human blood generally produced greater colorimetric readings for 100% hemolysis than did rabbit blood. Negro blood employed in two experiments early in this study failed to become 60% hemolyzed in 0.32% sodium chloride solutions, a salt concentration which in most instances seemed to completely lake erythrocytes from White donors. Eight additional blood samples from Negro volunteers were obtained and the osmotic fragility of the erythrocytes determined in various concentrations of sodium chloride. Although two of the ten Negro blood samples exhibited greater than 90% hemolysis in 0.32% sodium chloride solutions, seven samples showed less than 80% and three samples less than 60% hemolysis. An average of 74.7% hemolysis was shown by the ten Negro blood samples in 0.32% sodium chloride, the samples of Caucasian donors' blood (30 blood samples) employed in this study showed an average of 97.0% hemolysis in 0.32% sodium chloride solutions. Blood from Caucasians showed, on the average, 50% hemolysis in sodium chloride solutions of 0.381% concentration, the same degree of hemolysis did not occur in blood from Negroes until the sodium chloride concentration was decreased, on the average, to 0.342%. All of the Caucasian blood samples hemolyzed to a certain degree in 0.42% sodium chloride concentration whereas only four of the Negro blood samples exhibited hemolysis in this salt concentration.

Diseases of the blood and other pathological conditions have long been known to cause a variation in the osmotic fragility of erythrocytes (24). Sickle cell anemia, a disease occurring almost entirely in the Negro race, is characterized by sickle shaped erythrocytes and by an increase in the resistance of these cells toward hypotonic saline. In the present study, there was no evidence of sickling observed in the Negro blood samples.

SUMMARY

1. Hemolytic i values for various salts of gluconic acid have been determined and compared with i values calculated from freezing point depression data.

2. A procedure for the determination of the degree of hemolysis of erythrocytes suspended in colored aqueous solutions has been devised.

3. Using the hemolytic method, sodium and potassium gluconates gave i values which were approximately 25 per cent higher than those obtained by the freezing point depression method; this difference was only half as great when the hemolytic determinations were conducted in the presence of 0.2 per cent sodium chloride.

4. Magnesium and calcium gluconates gave higher i values by the hemolytic method than by the freezing point depression method when human erythrocytes were employed and lower ones when rabbit erythrocytes were used. The addition of 0.2 per cent sodium chloride raised the hemolytic i values of these salts when rabbit blood was used and lowered the values when human blood was used.

5. Manganese (II) gluconate gave slightly higher and cobalt (II) gluconate slightly lower i values from hemolytic data than from freezing point data.

6. The unusually low hemolytic i values obtained for iron (II) gluconate were, in the main, attributed to the instability of the solutions.

7. Zinc gluconate gave extremely high hemolytic i values due to the partial precipitation of the oxyhemoglobin liberated from laked erythrocytes making the colorimetric readings of the supernatant liquid misleading.

8. Erythrocytes from Negro donors were found to be, on the average, more resistant to osmotic hemolysis than those from Caucasian donors.

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Studies on the Adsorption of Odorous Materials II*

Surface Potential Changes Due to the Adsorption of Alcohol Vapors

By J. O. KOPPLIN†, J. R. EATON, and J. E. CHRISTIAN

The normal homologous alcohol vapors from 2- to 8-carbon-chain lengths were injected into an air stream in concentrations varying from 0.2 to 500 parts of alcohol vapor per million parts of air by volume and were passed over a water surface. Relationships between changes in the surface potential of the water surface and the chain length of the adsorbed alcohol molecules were determined and they were found to be analogous to the relationships between the olfactory intensities of the same alcohol vapors and the chain length of the vapors as reported by other research groups. The results of these tests suggest that the adsorption and surface potential change processes are influenced by the structure of the adsorbate molecule—a factor generally associated with odor.

IN A PREVIOUS PAPER (1), changes in surface potential caused by the adsorption of various vapors on solid or liquid adsorbing surfaces were discussed The various vapors in very low concentrations by volume were injected into an air stream which passed over the adsorbing surface The effects of changes in the humidity of the air stream and changes in the temperature of the adsorbing surface were presented along with a report on the application of radioactive tracer techniques for the measurement of the amount of vapor adsorbed.

In this paper the changes in the surface potential caused by the adsorption of several normal homologous alcohol vapors of a distilled-water surface are presented. These changes were determined by measuring the contact potential difference between two surfaces, using as a reference one surface the potential of which did not vary. The Kelvin method for measuring contact potential differences as modified by Zisman (2) was used in this investigation A description of the apparatus and general test procedure used is given in the preceding paper (1).

Because of the interest in the possible development of an objective instrument for the measurement of odor and because current opinion is that physical rather than chemical processes are critical in olfactory stimulation, the normal aliphatic homologous alcohols were studied as odorous air stream contaminants. Besides the fact that these alcohols can be smelled by the human nose, they are rather simple, well known organic substances concerning which considerable physical data are available—such as molecular weight, vapor pressure, dipole moment, and solubility.

EXPERIMENTATION AND DISCUSSION

Ethyl alcohol was adopted as a standard test vapor to check and to calibrate the system whenever a test was run or whenever the water surface was replaced. Except in a very few cases not more than three injections of any alcohol vapor were made into the air stream before the water surface was replaced. This was done because of the general loss in sensitivity of the surface with time and with repeated injections of contaminant vapor into the air stream. Surface sensitivity for a particular alcohol vapor in the air stream, as used in this paper, is measured by the ratio of the change in surface potential expressed in millivolts (mv.) to the alcohol vapor concentration in the air stream expressed in parts per million by volume (p p. m.).

For comparing the different alcohol vapors, measurement of the amount of vapor injected into the air stream was made in two different ways. The most often used measurement was in terms of parts per

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million by volume of alcohol vapor in the air stream. This was calculated using the product of two ratios—the ratio of the volume of saturated vapor injected into the air stream per unit of time to the volume of air passing over the surface per unit of time, and the ratio of the vapor pressure in mm. of mercury of the particular alcohol used to standard atmospheric pressure in mm. of mercury. Thus, the injection of two different alcohol vapors into the air stream at the same concentration in p. p. m. by volume required a greater volume of saturated vapor of the alcohol having the lower vapor pressure. The second method was the injection of the various saturated vapors at equal rates (ml. per second) into the air stream. This resulted in a smaller number of alcohol molecules in the air stream for each lower vapor pressure alcohol.

Ethyl Alcohol Vapor.—After approximately forty separate tests were run using different rates of injection of ethyl alcohol vapor into the air stream with different distilled water surfaces, the accumulated data were carefully analyzed. It was found from considering all of the first injections of ethyl alcohol vapor on each surface that the sensitivity of the individual water surfaces varied, but many water surfaces indicated good agreement in the mv. change in surface potential as caused by a certain number of p. p. m. by volume of ethyl alcohol vapor in the air stream. The change in surface potential was taken as the difference between the surface potential at the time injection of contaminant vapor into the air stream was started and the final equilibrium value of the surface potential with the contaminant laden air stream. If the surface potential were slowly drifting at the time the test was made, a correction was made to cancel the effect of the drift. Picking out various sets of data which were in close agreement as to the change in potential for a given vapor concentration, it was found that, if the mv. change in the surface potential were plotted against the logarithm of the alcohol vapor concentration in the air stream in p. p. m. by volume, a straight line relationship was indicated. This suggested the possibility of an exponential relationship between the p. p. m. of ethyl alcohol vapor in the air stream and the mv. change of the surface potential within the range tested. For ethyl alcohol the variation in p. p. m. was from 30 to 500.

As a check on this exponential relationship, a single distilled-water surface was used and ethyl alcohol vapor was injected six times—each succeeding time at an increased concentration in the air stream. As a check on the surface sensitivity, a seventh injection of ethyl alcohol vapor was made at the same concentration as the first injection. The difference in the change in surface potential caused by the first and last vapor injections was taken as a measure of the total loss in sensitivity of the water surface. It was expected that the loss in sensitivity between any two injections was proportional to the alcohol vapor concentration of the previous run. A correction was then applied for the loss in sensitivity to each of the last six test runs. The amount of correction applied to each change in surface potential was based on the previous injection concentration and calculated so that the change in surface potential for the last injection when corrected was the same as the first. When the p. p. m. *versus* the corrected mv. change in surface potential was plotted on semilog paper, the points were found to fall very close to a straight line.

Another check on the exponential relationship for ethyl alcohol was made using ethyl alcohol vapor along with varied concentrations of other alcohol vapors. In this case, a series of distilled-water surfaces was used. For each test the surface sensitivity of the particular water surface was standardized by determining the response to ethyl alcohol vapor of known concentration. This was done using the exponential relationship based on the most often encountered sensitivity of a distilled-water surface to ethyl alcohol vapor. Following this, a different alcohol vapor was injected and the change in surface potential for this second injection was corrected for the particular surface sensitivity as previously determined using ethyl alcohol vapor. Repeated tests were made in this manner at each of several different concentrations of the nonethyl alcohol vapor, and good agreement was found in the mv. change in surface potential caused by a given vapor concentration for several different alcohols. This indicated that the exponential relationship provided the correct factor for adjusting the sensitivity of individual water surfaces over the range of vapor concentration used so that all surface potential changes could be corrected for any differences in individual water surfaces.

The agreement found in both of the above checks concerning the correctness of the exponential relationship for ethyl alcohol was considered well within the accuracy of the equipment. Errors of 3% were expected in the determination of the flow rate of the air stream along with errors of 1% in the volume of vapor displaced into the air stream and errors of 3% in the determination of the vapor pressure. Errors were also expected in reading the surface potential, in determining the total change in surface potential (due to corrections for drift and other disturbances in the potential), and in calculating corrections for loss in sensitivity of various surfaces (due to exposure and variations within the surfaces themselves). These latter errors were estimated to be as great as 5%. The total experimental error was considered to be within 10%.

As a result of these tests, the exponential relationship between ethyl alcohol vapor concentration in p. p. m. by volume in the air stream and the mv. change in surface potential was accepted for the range of vapor concentrations employed, and the practice of rating distilled-water surfaces on the basis of the ethyl alcohol vapor sensitivity was considered valid.

Normal Homologous Alcohol Vapors.—Butyl alcohol at various concentrations was used as a contaminant vapor, and the exponential relationship between p. p. m. and mv. change in surface potential was again indicated. It was noted that plots of p. p. m. *versus* mv. change on semilog paper for both ethyl and butyl alcohol had practically identical slopes. See Fig. 1. The straight line relationship on semilog paper was then assumed for the other members of the normal homologous alcohol series, and checks were made at two different vapor concentrations for each different alcohol. The tests at each vapor concentration were repeated a minimum of three times to check agreement and reliability of the data.

In all, five different members of the homologous alcohol series were tested in the manner described above using distilled-water surfaces. These were

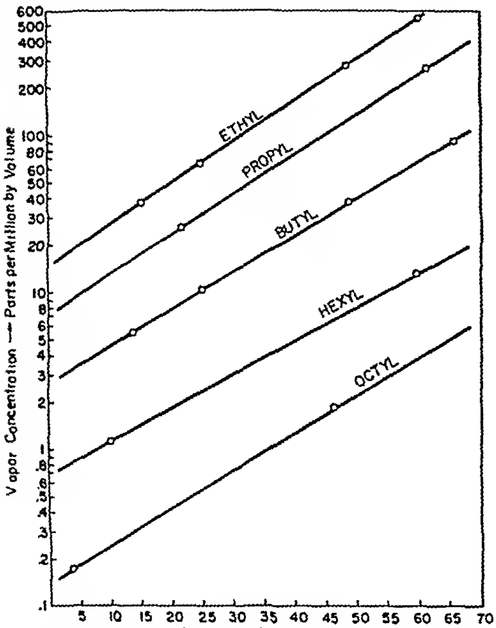

Fig. 1.—Relationship between surface potential change and vapor concentration for normal alcohol vapors.

ethyl, propyl, butyl, hexyl, and octyl alcohol having two, three, four, six, and eight carbon atoms, respectively. The straight line relationships between the logarithm of the vapor concentrations in p. p. m. by volume and surface potential changes in mv. for propyl, hexyl, and octyl alcohols were found, when plotted, to be very nearly parallel to the same lines plotted for ethyl and butyl alcohol vapors. The vapor pressure of ethyl alcohol is 64 mm. Hg at 27° while that of octyl alcohol is only 0.16 mm. Hg at 27°. This range in vapor pressure is greater than the maximum range over which saturated vapor could be injected into the air stream with the apparatus employed. The workable range for injecting saturated vapor into the air stream was from 0.015 to 0.25 cc. per second. This is a ratio of approximately 16:1 as compared with the ratio of 400:1 for the vapor pressures of ethyl and octyl alcohol at room temperature. Therefore it was not possible to inject vapors of all the different alcohols tested into the air stream at the same concentration in parts per million by volume. In order to increase the range over which comparisons could be made, some of the curves were slightly extended.

The data for ethyl, propyl, butyl, hexyl, and octyl alcohol vapors were considered and plotted in three different ways. The first was the relationship between the vapor concentration in the air stream in p. p. m. by volume and the mv. change in surface potential. The second was the relationship between the rate at which saturated vapor was injected into the air stream and the mv. change in surface potential. The third was the relationship between the vapor concentration in the air stream in p. p. m. by volume and the ratio of the mv. change in surface potential to the p. p. m. by volume vapor concentration.

The first relationship is shown in Fig. 1 where the logarithm of the vapor concentration in p. p. m. by volume is plotted against the change in surface potential. This figure indicates that, for a given change in the surface potential, the required p. p. m. decreased as longer-chain alcohol vapors were used. The lines for the various alcohols are nearly parallel and each is well separated from the next. The various lines could be represented by an exponential equation of the form $y = a(b)^x$, where y refers to the p. p. m. and x to the mv. change in surface potential. The value of b is approximately 1.0 in each case and the various values of a are: ethyl, 17.18; propyl, 6.76; butyl, 3.01; hexyl, 0.65; and octyl 0.15.

The second relationship, which was between the rate in ml. per second at which saturated vapor was injected into the air stream and the mv. change in surface potential, is shown in Fig. 2. The reason for the interest in this relationship was that many olfactory studies had been done with trained investigators sniffing equal amounts of saturated vapor. The curves appear to be logical in that repeated increases in the injection rate produced successively smaller increases in the mv. change of the surface potential. The curves generally indicate a smaller change in the surface potential for a given injection rate for each heavier alcohol vapor. The unusual feature of Fig. 2 is the location of the butyl line which indicates that butyl alcohol vapor caused a greater change in surface potential than either ethyl or propyl for a given injection rate of saturated vapor. This might be due to the greater solubility of ethyl and propyl alcohol in water which reduced the ability of these vapors to change the surface potential of the water.

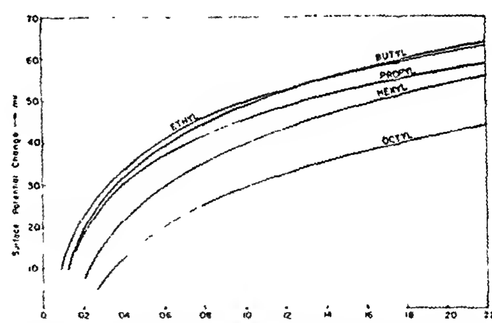

Fig. 2.—Relationship between surface potential change and injection rate of saturated vapor for normal alcohol vapors.

The third relationship considered was between the vapor concentration in the air stream in p. p. m. by volume and the ratio of the change in surface potential to the vapor concentration. This relationship for the various alcohols is shown in Fig. 3. The mv. to p. p. m. ratio[1] can be considered as a measure of the ability of a given alcohol vapor to change the surface potential upon adsorption, and the graph shows that this ability increases with increased chain length.

The hydroxyl group of the normal alcohol molecules is water attractive and thus it is this end of the

[1] This ratio has also been defined as surface sensitivity when the ratio is used to describe the sensitivities of various surfaces to a given contaminant vapor.

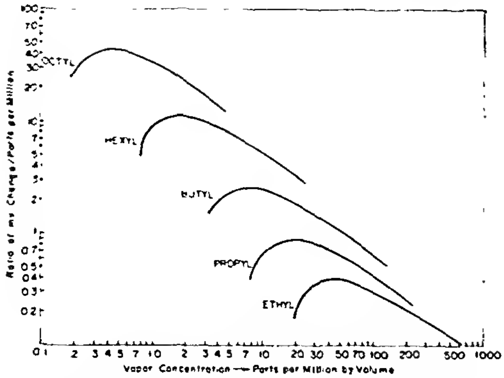

Fig. 3.—Relationship between vapor concentration and ratio of mv. change to p. p. m. for normal alcohol vapors.

chain which attaches itself to the water surface (3). Such groups attached to a hydrocarbon-chain might be called water-soluble groups, and in the shorter-chain compounds, such as ethyl alcohol, the water-soluble hydroxyl group confers solubility on the whole molecule. In the long-chain compounds, such as the heavier alcohols, this group cannot pull the whole molecule into the water owing to the resistance of the long-chains to immersion, and thus the adsorbed layer is made up of molecules with the water-soluble group down close to the water and the hydrocarbon-chain extending away from the surface. The extent of the attraction for water of the water-soluble groups can be roughly determined from the length of the carbon-chain required to prevent solution of the molecule.

Figures 1 and 2 indicate that, for a given concentration of alcohol vapor, the longer the chain length, the greater the change in surface potential. The reason the longer-chain alcohols appear to have a greater effect on the surface potential may be due to the fact that they are insoluble in water and must remain on the surface when adsorbed. The nearly complete recovery of the surface potential following the termination of an injection of ethyl alcohol vapor into the air stream probably was due not only to desorption from the surface but also to the movement of the adsorbed alcohol molecules from the surface to the interior of the liquid. The amount of alcohol going into solution with the water was not judged sufficient to cause any permanent change in the surface potential. The changes in surface potential caused by the different alcohol vapors most likely were due to the vertical component of the dipole of the adsorbed molecules with any reorientation of the water molecules playing a minor role. Thus the difference in the ability between short- and long-chain alcohol molecules to change the surface potential of a water surface may be mostly due to orientation of the adsorbed molecules on the surface, with the longer-chain molecules in better alignment.

OLFACTORY COMPARISONS

An interesting comparison can be made between the data secured using the normal homologous alcohol vapors and data obtained by other investigators who studied the olfactory properties of the same alcohol vapors. Various homologous series, and in particular the alcohols, have been investigated in recent years with regard to the olfactory intensities of these vapors.

Kruger, Feldzamen, and Miles (4) of Yale University used three people especially trained in olfactory observation to determine the olfactory intensities of ten aliphatic alcohols from 3 to 12 carbon-chain lengths. Some of the stated conclusions of their study were that: (*a*) the alcohols which are soluble in both polar and nonpolar solvents were found to have the more intense odors; and, (*b*) within most of the range of alcohols tested, olfactory intensity decreases with increasing chain length unless correction is made for the different vapor pressures, in which case the trend is reversed. This is interpreted to imply that olfactory intensity decreases with increasing chain length when the alcohol vapors were tested using specific dilutions of saturated vapor, but for equal molecular concentrations, the olfactory intensity increased with increasing chain length.

The conclusion of the Yale group with regard to chain length is analogous to the relationship between chain length and surface potential change found in this research. Comparisons of the various alcohol vapors in terms of equal p. p. m. by volume indicates that the change in surface potential increases with increasing chain length, as shown in Fig. 1. It is also interesting to make a comparison between the two different studies in terms of equal volumes of saturated vapor. In Fig. 4 the change in surface potential is plotted against chain length for the specific injection rate of 0.2 ml. per second of saturated vapor. This is similar to a plot of odor intensity *versus* chain length as determined by each of the three "sniffers" used by the Yale group. In general, the "sniffers" rated the alcohols of 4, 5, and 6 carbon members slightly more intense than either the lighter or heavier alcohol vapors; however, the individuality of human beings was also apparent.

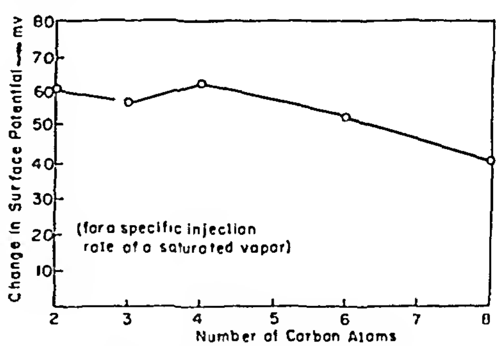

Fig. 4.—Change in surface potential *versus* chain-length for normal alcohol vapors.

The olfactory properties of the normal homologous alcohols were also studied by Dethier and Yost (5) of Johns Hopkins University. This group experimented with blowflies to make use of several advantages offered by insects as compared to man for this type of study. The general procedure used by them was to pass two air streams with little or no mixing through a cage containing the flies and to increase the alcohol vapor concentration in one air stream until the flies rejected it in preference to the other. They reported that when rejection thresh-

olds of the normal alcohols were expressed as molar concentrations and plotted against their respective chain lengths on logarithmic coordinates, the trend toward linearity was at once apparent.

Using the data secured in this investigation and arbitrarily taking a 25 mv. change in the surface potential as a critical or rejection figure, a graph as shown in Fig. 5 was obtained. The logarithmic coordinates are chain length and the alcohol concentration in p. p. m. by volume which produced the 25 mv. change in surface potential. The relationship between the logarithm of the vapor concentration and the logarithm of the chain length is clearly linear, and comparison of this relationship with the relationship reported by Dethier and Yost indicates a definite similarity.

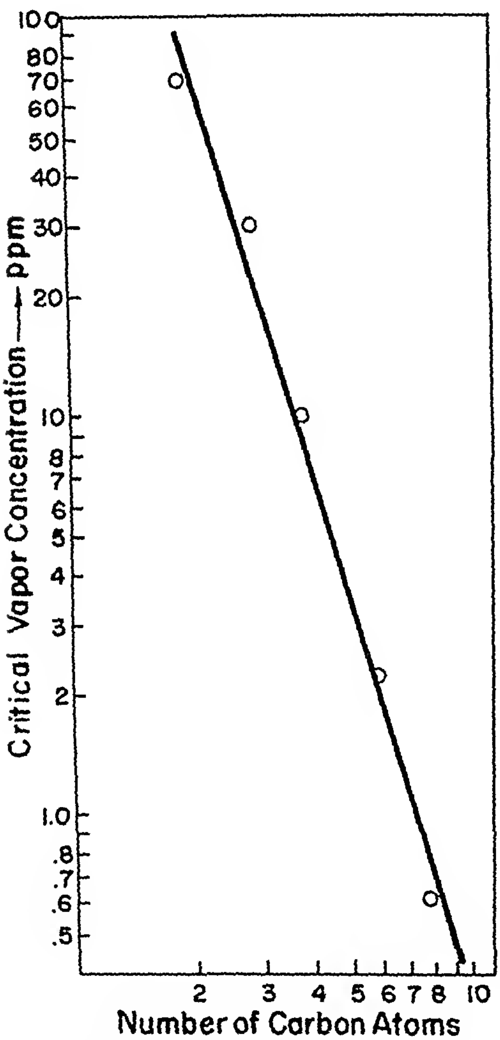

Fig. 5—Vapor concentration for a specific surface potential change *versus* chain-length for normal alcohol vapors.

SUMMARY

The general results of this study are:

1. Equal concentrations of different vapors of a homologous alcohol series in an air stream, measured in parts per million by volume, cause different changes in the surface potential of a distilled-water surface.

2. The presence of alcohol vapors in an air stream at concentrations of the same order of magnitude as the minimum concentrations detectable by the human nose can be determined by measurement of surface potential change.

3. Relationships between changes in surface potential and chain length of the normal homologous alcohols are analogous in certain respects to reported relationships between olfactory stimulation and chain length of the same vapors.

CONCLUSIONS

There appear to be several factors associated with the surface potential change of a water surface caused by adsorption which may be advantageous as far as the detection of odorous materials in the atmosphere is concerned. These are: (*a*) the facility with which a liquid can be employed; (*b*) the capability of a liquid to act as a solvent—as solubility has often been associated with odor (6); and (*c*) the orientation freedom of both the liquid and adsorbate molecules. These factors allow the adsorption and surface potential change processes of a liquid surface to be more readily influenced by the architectural arrangement of the adsorbate molecule, a factor generally considered in the study of odor. The results of this investigation indicate certain analogies between the changes in surface potential and the olfactory stimulation produced by certain vapors but should not be interpreted to imply that odor has been measured by electrical-mechanical means. The results do indicate the desirability of further research regarding surface phenomena with the goal of learning more about the roles played by the various physical properties of the adsorbate molecules.

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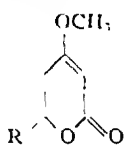
The Reaction of Δ^2-Cyclohexenone With Trifluoroperoxyacetic Acid*

By EDWARD E. SMISSMAN and FRED B. BLOCK

The reaction of 2-cyclohexenone with trifluoroperoxyacetic acid gave 2-hydroxyadipic acid as a final product. The failure of 2-cyclohexenone to yield Δ^2-caprolactone or 2,3-dihydroxycaprolactone demonstrates the favored transition state involves carbon-carbon double bond participation and not alkyl group migration.

THE ONLY reported synthesis of dihydrokavain [6-(β-phenylethyl)-5,6-dihydro-4-methoxy-2-pyrone], IA, a constituent of *Piper methysticum*, is the reduction of the closely related compound kavain IB, (6-styryl-5,6-dihydro-4-methoxy-2-pyrone) (1,2)



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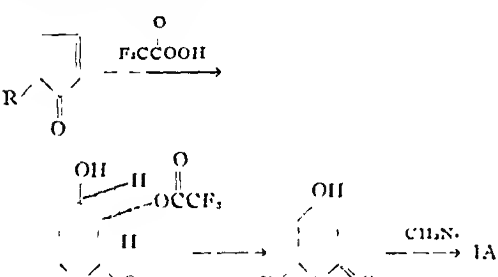
(*A*) R = $C_6H_5CH_2CH_2$—
(*B*) R = $C_6H_5CH{=}CH$—

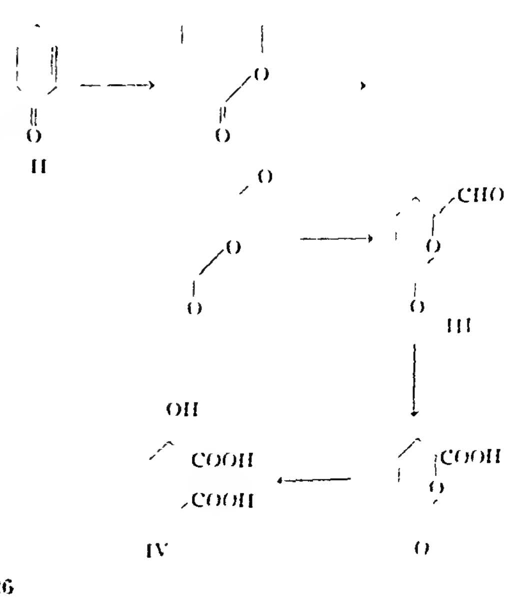
A possible synthetic route to dihydrokavain, utilizing the Baeyer-Villiger reaction can be outlined as follows:



The Baeyer-Villiger lactonization and hydroxylation step appeared to be of particular interest, as this one-step combination of reactions had not been reported. A good method for preparing 5-alkyl-2-cyclopentenones, as well as 2-cyclopentenone itself, could not be found and rather than attack this portion of the problem, it was decided to use 2-cyclohexenone, II, as the model compound to test the practicality of the proposed reaction.

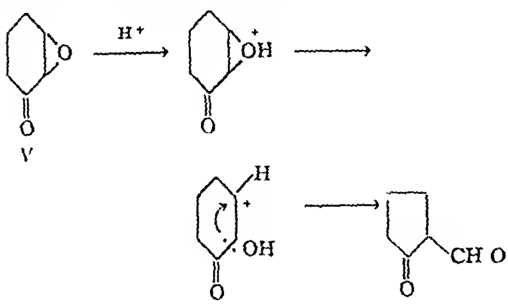
Peroxytrifluoroacetic acid was selected as the reagent for the rearrangement-hydroxylation step because of high yields reported in the preparation of lactones (3) from cyclohexanone and cyclopentanone (71 and 84 per cent, respectively) and the hydroxylation of normally resistant acrylates and methacrylates in good yield by this reagent (4). The reaction procedure utilized gave an amount of nonvolatile material in excess of that expected for the peracid oxidation. The infrared absorption spectrum of the reaction mixture at this point indicated the possible presence of a trifluoroacetyl group, 5.59 μ (5). The product was treated with 3 per cent methanolic hydrogen chloride to effect methanolysis, and on removal of solvent a red oil exhibiting infrared bands at 5.59 and 5.76 μ, in addition to a band at 2.84 μ, was obtained. The presence of a moderately strong band at 5.59 μ was unexpected as this band was originally believed associated with the trifluoroacetoxy group.

Vapor phase and column chromatography would not resolve this red oil and it was decided to make a solid derivative. On treatment with *p*-toluidine, a white crystalline compound was obtained and had the correct analysis for a hydroxyadipic acid di-*p*-toluidide. The presence of the adipic acid skeleton was demonstrated by the isolation of adipic acid after the red oil was refluxed with hydriodic acid, while the presence of the hydroxyl group was positively confirmed by the conversion of the di-*p*-toluidide derivative to its acetate. The location of the hydroxy group was suggested by the apparent course of the reaction to be in the 2-position, IV.



* Received April 3, 1959, from the University of Wisconsin, School of Pharmacy, Madison 6.

Synthetic 2-hydroxy adipic acid was prepared by an independent method and its di-p-toluidide was identical with the material obtained from the reaction mixture. A small amount of a 2,4-dinitrophenylhydrazone could be obtained from the reaction mixture and it corresponded to 5-formyl-5-pentanolid, III. Work by House and Wasson (6) on the rearrangement of α,β-epoxycyclohexanone has demonstrated that under acidic conditions 2,3-epoxycyclohexanone, V, rearranges with ring contraction to the keto



V VI

aldehyde, VI. The failure of 2-cyclohexenone on peroxidation to yield Δ^2-caprolactone or 2,3-dihydroxy caprolactone demonstrates the transition state favored in this compound involves carbon-carbon double bond participation and not alkyl group migration.

EXPERIMENTAL

Reaction of 2-Cyclohexenone with Peroxytrifluoroacetic Acid.—Seventy-five milliliters of dry methylene chloride and 11.8 Gm. (0.312 mole) of 90% hydrogen peroxide were placed in a flask equipped with a thermometer, dropping funnel condenser, and magnetic stirrer. Over a period of one hour, 78.5 Gm. (0.374 mole) of trifluoroacetic anhydride was added to the rapidly stirred mixture which was cooled in an ice bath. Additional stirring was continued for fifteen minutes. To the cooled (10° or below) peroxytrifluoroacetic acid was added 10.0 Gm. (0.104 mole) of 2-cyclohexenone, dissolved in 25 ml. of dry methylene chloride, over a period of one and one-half hours. After addition was complete the red solution was allowed to warm to room temperature and then refluxed for two hours. The volatile solvents were removed under reduced pressure.

The liquid residue (24.3 Gm.) was refluxed with 300 ml. of 3% methanolic hydrogen chloride for three hours and then allowed to stand eight hours at room temperature. The volatile material was removed *in vacuo* leaving 15.5 Gm. of liquid residue which had an infrared spectrum showing bands at 2.84, 5.59, and 5.76 μ, ultraviolet spectrum showed only end absorption.

Isolation of Adipic Acid. To 0.947 Gm. (0.005 mole based on dimethyl 2-hydroxy adipate) of liquid residue, under a constant stream of dry nitrogen, was added 17 Gm. of freshly distilled 57% hydriodic acid. The mixture was refluxed one hour and then concentrated to a small volume. The mixture was dissolved in ether, washed with sulfurous acid, and dried. A 66.6% yield, 0.488 Gm. (0.0033 mole) of adipic acid was obtained, m. p. 150.7–152°.

2-Hydroxy Adipic Acid Di-p-toluidide.—A crystalline derivative was prepared by combining 0.202 Gm. (0.001 mole based on dimethyl-2-hydroxyadipate) of the liquid residue with 0.814 Gm. (0.0076 mole) of p-toluidine and heating the mixture at 190–200° for two hours. After cooling and washing with ether, 2-hydroxyadipic acid di-p-toluidide, m. p. 183.5–188.5° was obtained. It was recrystallized from ethanol-water, m. p. 187.4–187.9°.

Anal.—Calcd. for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.65; H, 7.23; N, 8.03.

2-Acetoxyadipic Acid Di-p-toluidide. Forty-five milligrams of the di-p-toluidide of 2-hydroxyadipic acid was dissolved in 0.7 ml. of pyridine and 0.5 ml. of acetic anhydride was added; the mixture was heated for one hour. The excess reagents were removed *in vacuo* leaving a solid product which was recrystallized from ethanol-water, m. p. 172.4–173.2°.

Anal.—Calcd. for $C_{22}H_{26}N_2O_4$: C, 69.09; H, 6.85; N, 7.33; acetyl, 11.25. Found: C, 69.19; H, 6.56; N, 7.26; acetyl, 13.3.

2-Hydroxyadipic Acid.—Dimethyl-2-chloroadipate, b. p. 145–150°/15 mm., N_D^{20} 1.4505, (lit., b. p. 138–139°/10 mm., N_D^{20} 1.4510) (7) was prepared by the method of Triebs and Holbe.

One gram of dimethyl-2-chloroadipate was treated with 13 ml. of 10% aqueous methanolic sodium hydroxide. After three and one-half hours of refluxing, the mixture was cooled, acidified with concentrated hydrochloric acid, and evaporated to dryness. The solid residue was boiled with ether and the ether solution evaporated to give a colorless oil which crystallized on standing. This material was converted to the di-p-toluidide, m. p. 170.8–172.5°. Mixed melting points with previously described samples gave no depression.

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16α-Hydroxy Steroids II*

Partition Chromatography of Triamcinolone and Related Steroids

By LELAND L. SMITH, THEODORE FOELL, RALPH DE MAIO, and MURRAY HALWER

Paper chromatographic systems and column partition systems suitable for analyzing triamcinolone in pharmaceutical preparations and in extracts of biological samples are described. Comparative mobilities of related steroids are reported for the several systems. Both qualitative and quantitative aspects of the chromatographic operations are described.

The characterization of the corticosteroid hormone triamcinolone (9α fluoro-11β,16α,17α,21 - tetrahydroxy - 1,4 - pregnadiene - 3,20 dione) by instrumental means has been reported in the first paper of this series (1). For analyses involving more complex situations as exist in metabolic studies of the drug preliminary chromatographic separations become necessary. The several paper chromatographic systems and column partition systems which have been of use in routine examination of triamcinolone and related steroids are presented in this paper.

PAPER CHROMATOGRAPHY, QUALITATIVE

Recently reports have been published covering the use of Zaffaroni type paper chromatographic systems for the separation of more polar steroids (2), which systems together with the chloroform/formamide system of Schindler and Reichstein (3) afford separation of a variety of more polar steroids. The application of Zaffaroni type systems to triamcinolone and related 16α-hydroxylated steroids has been described (4). Although some Bush-type systems capable of rapid separations of more polar steroids have been described (5), no systematic examination of very polar steroids in Bush type systems has been reported. In our hands modified Bush type systems have been found to be more versatile in the complex separations involved in the production and analysis of triamcinolone than have the Zaffaroni type systems.[1]

For the routine examination of triamcinolone samples derived from a variety of sources the following conditions must be met: (a) that the $\Delta^{1,4}$ 3-keto steroids be well separated from their respective Δ^4 3 ketosteroid analogs, (b) that the respective rearrangement product of each 16α hydroxylated steroid be well separated from the parent steroid, and (c) that running time be limited to no longer than overnight. The six solvent systems used are described in the experimental part. Where possible the systems have been adjusted to afford mobilities for at least one steroid component of interest in the vicinity of R_f 0.5. A more stringent requirement met by systems I and II for the free steroid alcohols is of importance, namely that the rearrangement product 1,2 dihydrotriamcinolone isomer be separated from triamcinolone. The fortuitous similarity of papergram mobilities in several Bush-type systems of these two steroids is illustrated in Table I with systems III and IV.

Using the six systems described in descending chromatography it is possible to separate many derivatives of triamcinolone and many intermediates involved in the several reported syntheses (6, 7). Examination of the free steroid alcohols is made in systems I through IV, with system II being used for general qualitative examinations, system I run overnight for quantitative analysis. Systems III and IV serve for both free alcohols and their acetates, etc., while systems V and VI are for the acetates and acetonide derivatives.

An arbitrary equilibration period of two hours is used for each papergram system, and the tanks are maintained at about 29° in a closed room. Although higher temperatures and longer equilibration periods have been examined, the short running time and the lower temperature afford useful chromatographic separations. The 29° temperature is maintained by one 100 watt incandescent lamp overhead in a small tank room. Using these conditions the data of Table I were collected. Although it is possible to obtain reproducible R_f values for these steroids, standard steroid markers are run with each set of samples, as several factors influence mobility under the described conditions.

It is of particular importance in the case of system II that attention be paid to the variability in R_f values. This system is of great value in examination of triamcinolone samples, however, the mobility of a steroid is dependent on the actual running time. Thus, at one hour lower R_f values are obtained for all steroids studied than at the standard four hour running time. Also the solvent front migration rate decreases with increasing running time, covering 17.5 cm in the first hour, 14.3 cm in the second hour, and 11.0 cm in the fourth hour. Freshly prepared tanks yield mobility values as presented in Table I, however, as the tanks are used, slight losses in solvent plus other effects cause a decrease in mobility of all steroids, so that after several weeks of use, lower R_f values are generally obtained. Despite these several deficiencies, system II is of very great value in studies of triamcinolone chemistry and biochemistry. It is possible that the characteristics mentioned obtain with other Bush systems described in the literature.

The detection of the steroids on papergrams is accomplished with several techniques. The ultraviolet absorption properties of these steroids affords a ready means of location using photographic meth-

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[1] The mobility of triamcinolone in the chloroform/formamide (50% methanolic formamide impregnation) system is 3.1 cm./22 hr., of 1,2 dihydrotriamcinolone, 3.5 cm./22 hr., of 9α fluorohydrocortisone, 20 cm./22 hr.

TABLE I.—PAPERGRAM CHARACTERIZATION OF TRIAMCINOLONE AND RELATED STEROIDS

Steroid	R_f of Free Alcohol I	II	III	IV	R_f of Acetates, Diacetates IV	V	VI	R_f of 16α, 17α-Acetonide IV	V	VI
Triamcinolone	0.18	0.42	0.14	0.13	0.89	0.27	0.12	0.88	0.36	0.13
1,2-Dihydrotriamcinolone	0.24	0.52	0.22	0.19	0.92	0.40	0.22	0.92	0.46	0.25
9α-Fluoroprednisolone	0.37	0.67	0.37	..	0.89	0.30	0.12	..	..	..
9α-Fluorohydrocortisone	0.45	0 80	0.47	0 59	0.90	0.35	0.18	..	..	..
Triamcinolone isomer	0.10	0.22	0.08	0.06	0.76	0.08	0.02	0.62[a]	0.10[a]	0.03[a]
1,2-Dihydrotriamcinolone isomer	0.12	0.30	0.13	0.10	0.85	0.18	0.06	..	0.16[a]	0.06[a]
16α-Hydroxyprednisolone	..	0.44	..	0.23	..	..	..	..	0 44	0.29
16α-Hydroxyhydrocortisone	..	0.49	..	0.30	..	..	..	..	0.57	0.40
Prednisolone	0.53	0.73	0.46	0.44	0.89	0.30	0.15	..	..	..
Hydrocortisone	0.58	0.75	0.53	0.53	0.93	0.46	..	..	..	..
Prednisone	0.69	0.79	0.58	0.66	..	0.60	.	..	..	..
Cortisone	0.73	0.81	0.65	0.69	..	0.66	0.43	..	..	..

[a] The acetonides are in all probability 16α, 17aα-cyclic acetonides of the D-homo-16α,17aα-dihydroxy-17aβ-hydroxymethyl-17-ketones and not simple 16α,17α-acetonides.

ods (8) or using fluorescent screens (9). The reducing properties of triamcinolone permit facile detection using tetrazolium salts, or Tollen's reagent.

The failure of triamcinolone and related $\Delta^{1,4}$-3-ketosteroids to give the alkaline fluorescence test of Bush (10) which is specific for Δ^4-3-ketones, allows direct differentiation of these types. Although *p*-phenylenediamine phthalate (11), 2,4-dinitrophenylhydrazine (12), and salicyloyl hydrazide (13) have been variously reported as color tests for $\Delta^{1,4}$-3-ketosteroids, in our hands these tests have not been of use with triamcinolone and other common $\Delta^{1,4}$-3-ketones. However, isonicotinic acid hydrazide has proven an excellent method for the detection of both $\Delta^{1,4}$-3-ketosteroids and Δ^4-3-ketosteroids on papergrams (14).

Our routine examination of paper chromatograms involves five steps: (*a*) examination under short wavelength ultraviolet light using a modified Haines-Drake scanner (9) as described by von Arx and Neher (15), (*b*) examination under long wavelength ultraviolet light (365 mμ) for fluorescent components, (*c*) application of the weak ethanolic isonicotinic acid hydrazide reagent of Smith and Foell (14) for the detection of Δ^4-3-ketosteroids, followed by (*d*) application of the strong methanolic isonicotinic acid hydrazide reagent for $\Delta^{1,4}$-3-ketones, and finally (*e*) alkaline tetrazolium blue spray for reducing α-ketols. The specificity conferred by this series of tests enables an experienced operator to establish the probable steroid nature of an unknown component, and together with relative mobility measurements, to recognize a large variety of metabolites and alteration products of triamcinolone and related steroids.

Experimental

The paper partition systems are prepared in the usual manner (10) by equilibrating appropriate volumes of the solvents of choice for several days at the temperature at which the solvent mixture will be used. In practice, the mixtures are kept in the same room as the chromatographic jars and the phases are separated a short time before the chromatographic run is to be started. The upper (organic) phase is used for irrigation of the papers (mobile phase) in all systems. The compositions of the solvent systems used are as follows:

System I: benzene/ethanol/water, 2/1/1. Equilibration time, two hours, running time, sixteen hours. (R_f data were taken on the system run for four hours.) For free steroid alcohols.

System II: benzene/acetone/water, 2/1/2. Equilibration time, two hours, running time, four hours. For free steroid alcohols.

System III: benzene/dioxane/water/acetic acid, 4/1/2/1. Equilibration time, two hours, running time, five hours. For free alcohols and acetates.[2]

System IV: benzene/ethanol/water, 2/1/2. Equilibration time, two hours, running time, five hours. For free alcohols and acetates.

System V: toluene/petroleum ether (b.p. 30–60°)/methanol/water, 12/8/13/7. Equilibration time, two hours, running time, two and one-half hours. For acetates, acetonides, etc.

System VI: benzene/petroleum ether (b. p. 90–100°)/methanol/water, 5/5/7/3. Equilibration time, two hours, running time, three and one-half hours. For acetates, acetonides, etc.

These solvent systems were all run in the descending technique, using Whatman No. 1 filter paper.

PAPER CHROMATOGRAPHY, QUANTITATIVE

For quantitative analysis the separated components are eluted and ultraviolet absorption measurements are made on the eluates. System I is recommended for this work, as the system has the designed advantage of running overnight so sample preparation, equilibration, etc., may occur in the afternoon of one day and location, elution, and spectrophotometric analysis be completed the next day.

Experimental

Preparation of Tanks.—Glass cylinders 12 inches in diameter, 24 inches high are lined with Whatman No. 1 filter paper, the liners dipping into the stationary phase in the bottom of the tank. A dish of mobile phase is placed in the bottom of the tank and a filter paper wick is suspended from the solvent trough apparatus in the center of the tank so that the wick dips into the dish of mobile phase. Lids are ground to fit each tank individually and are held solvent tight by plastic tubing rings filled with lead shot. The lids are not lubricated. After forty-eight hours of equilibration at 29° the tank is ready for use.

[2] This solvent system was suggested by Mr. R. H. Blank of these laboratories.

Preparation of Papers.—Whatman No. 1 filter paper (18.25 × 22.5 in.) is washed chromatographically with ethanol for seventy-two hours. After the papers are washed they are handled only with tissue papers. The dried (hood) papers are cut into sheets 9.5 cm. wide, a starting line is ruled across the paper four inches from one end and the other end is serrated with pinking shears to allow free runoff of excess solvent, and the papers are stored in filter paper wrappers until used.

Sample Preparation.—The steroid sample is weighed on a Sartorius Micro Torsion Balance, Model MTB-1, to the nearest 0.02 mg. Generally a 1 mg./ml. solution is prepared in distilled methanol. Application of the sample is made via a 100 μl. micropipet calibrated "to contain" (a later improvement was made when "to deliver" pipets became available commercially). Care is taken that the steroid solution is not drawn above the calibration mark. Application of the solution to the paper is made by streaking across the paper; the streak is begun about 1 cm. from one edge and terminated about 1 cm. from the opposite edge, such application resulting in a decreased "edge" effect in the band movements. The "to contain" pipet is then washed out with 100 μl. of methanol, the washings being applied to the paper in a similar manner. Suitable drying time is allowed between applications. Samples are prepared in duplicate, using the same pipets and solutions. The prepared papers are then hung in the chromatographic tank for equilibration.

Chromatographic Development.—Chromatographic tanks for quantitative papergram assay are kept separated from tanks prepared in an identical manner but used for qualitative papergram examinations. The tanks prepared using system 1 are equilibrated for two hours after the prepared papers are placed in them. The running time (sixteen hours) is the same as used for qualitative examination, unless some of the more mobile components (such as 9α-fluorohydrocortisone) are to be assayed, in which case suitable shorter times are required. Blank papergrams containing no steroid but otherwise handled in exactly the same way as the sample sheets are run with each tank used; thus three sample sheets and one blank sheet are generally run together in one tank. The developed papers are dried in air for thirty minutes, viewed rapidly under the modified Haines-Drake ultraviolet scanner, and the steroid-absorbing zones marked in pencil. In order to see steroids on the papergram with this technique at least 8–10 μg. of steroid is required. Where the amount of steroid per zone is less than this limit it is necessary to determine the position which would be occupied by the steroid sought from relative mobility measurements. Our practice has been to measure all mobilities in terms of triamcinolone as unit mobility (R_t 1.00); thus triamcinolone isomer has R_t 0.54; 1,2-dihydrotriamcinolone R_t 1.30, 1,2-dihydrotriamcinolone isomer, R_t 0.74, etc. The zones located via ultraviolet absorption are cut out; the zones determined by calculation are cut out 1.5 in. wide. The pieces are warmed with 10 ml. of absolute ethanol for one hour at 60° in closed tubes, cooled to room temperature, and the absorbance of the solutions determined (after centrifugation, if necessary to remove lint) at 239 mμ read against their appropriate blanks treated similarly.

The blank sheet is cut into zones exactly according to the zones found for the steroids being assayed, eluted in the same way, and read against absolute ethanol as a check on the operation, and then used as the blank solution for the appropriate steroid determination. The blank absorbance values are of the order 0.002 to 0.03 units *versus* absolute ethanol.

Calculation of Results.—A spectrophotometric factor (μg./ml. divided by absorbance at 239 mμ) is determined on a pure steroid sample (not chromatographed). The absorbance of the unknown eluate times the factor times the eluate volume gives the micrograms of steroid found, uncorrected for recovery. The factors used are: triamcinolone, 26.8; 1,2-dihydrotriamcinolone, 23.7; triamcinolone isomer, 26.8; 1,2-dihydrotriamcinolone isomer, 23.7; 9α-fluorohydrocortisone, 24.9.

In a series of experiments with pure steroids an average recovery of 80 μg. out of 100 μg. applied to the paper was found. The factor was the same over the range 25–100 μg. of sample applied, although at levels less than 25 μg. somewhat less (*ca.* 70%) was found. Individual recovery factors on pure samples were often as high as 90%. Recovery from mixtures of several components was not affected by the nature of the mixture. The recovery factor should be determined under the conditions used and applied to the uncorrected data.

Alternate treatment in the form of calibration curves constructed over the range 0–100 μg. of steroid applied *versus* μg. of steroid recovered yield linear relationships, although irregularities occur in individual cases. Attempts to rely on the absolute weight of steroid found for the calculation of composition were not pleasing, so sample composition is expressed in terms of per cent of total steroids found, with the actual weight found also reported. Where material balances for multicomponent mixtures fell much below the 80% factor the assay is suspect.

Some representative analyses are presented in Table II. All samples are run in duplicate and are so reported.

TABLE II.—REPRESENTATIVE DUPLICATE ANALYSES ON TRIAMCINOLONE PROCESS SAMPLES

Sample No.	Triamcinolone μg.[a]	Triamcinolone %[b]	Triamcinolone Isomer μg.[a]	Triamcinolone Isomer %[b]	1,2-Dihydrotriamcinolone μg.[a]	1,2-Dihydrotriamcinolone %[b]
1	82.5	98.4	1.4	1.6	0	0
	83.9	100.0	0	0	0	0
2	79.1	97.5	1.1	1.4	0.9	1.1
	80.7	94.7	2.1	2.5	2.4	2.8
3	71.0	91.5	2.7	3.6	1.4	1.9
	71.0	93.7	2.9	3.8	1.9	2.5
4	84.1	94.1	0.8	0.9	4.5	5.0
	83.4	93.4	1.3	1.5	4.5	5.1
5	45.4	50.1	45.1	49.9	0	0
	44.3	49.5	45.1	50.5	0	0

(Steroid Found)

[a] μg. found, uncorrected for recovery.
[b] Per cent of total spectrophotometric activity found.

COLUMN PARTITION CHROMATOGRAPHY

For such purposes as final analysis of steroid samples for commercial sale the quantitative analytical methods involving paper chromatography often are unsatisfactory and methods employing column

chromatography are used. The increased sample charge permits more precision and accuracy in the measurement of minor contaminants, indeed, permits multiple analyses to be made on each resolved fraction. Although somewhat lower resolving power is usually had in comparison with paper chromatographic separations using the same solvent mixtures, the advantages of rapid operation, increased by the use of air pressure, together with the possibility of application of several specific methods to the eluates and increased precision, etc., makes this technique invaluable where several closely related steroidal impurities must be controlled.

A partition system devised by Dr. N. Rigler, of these laboratories, comprised of dioxane/cyclohexane/water in varying proportions, has proved of analytical as well as of preparative value. According to the degree of resolution desired the solvent ratios of 5/2/1, 5/3/1, and 5/4/1 may be used. Characteristic hold-back or retention volumes permit rapid recognition of the identity of the steroids present in a given sample, and by the use of modern detecting devices, ultraviolet absorbing fractions may be collected separately one from the other and analyzed according to the need.

Experimental

Preparation of Solvent.—1,4-Dioxane should be freshly distilled from over potassium hydroxide pellets. Reagent grade cyclohexane is used without prior distillation. The solvents, dioxane/cyclohexane/water, are mixed in the proportions 5/2/1, 5/3/1, or 5/4/1 according to the degree of separation required, thoroughly equilibrated at the temperature at which the column is to be run (room temperature), and allowed to separate.

Preparation of Column.—Celite 545 diatomaceous earth is washed with methanol and with ethyl acetate to free it of impurities absorbing in the 240 mμ region. To 4.5 Gm. of the washed Celite diatomaceous earth is added 1.9 ml. of the separated lower phase of the solvent system to be used, and thorough mixing of the solvent throughout the support is effected with a spatula. The moistened support is packed into a glass column, 1 cm. in inside diameter and 35 cm. long (exclusive of a 125-ml. reservoir at the top), in layers of about 0.5 Gm. Each increment is packed uniformly by repeated light strokes with a ramrod whose diameter is just slightly less than that of the column. Uniform packing is essential for proper resolution and for reproducible hold-back volumes.

Sample Preparation.—Steroid samples from fermentation broths, etc., by extraction with ethyl acetate or other appropriate solvent are evaporated to dryness without heat and the residue is dissolved in the lower phase of the solvent mixture in the same manner as for crystalline samples. Sufficient broth extract must be taken to afford about 3 mg. of total steroids. Approximately 3 mg. of the steroid sample is dissolved in 0.23 ml. of the lower phase of the appropriate solvent mixture and thoroughly mixed with 0.45 Gm. of washed Celite 545 diatomaceous earth support. The mixture is packed on top of the prepared column. Ten milliliters of the upper phase of the solvent mixture is used to rinse the dish in which the sample has been prepared and the rinse is added to the top of the packed column and the liquid level is marked. Air or nitrogen pressure is applied at 3–5 lb./sq. in. until the first drop of eluate appears. The level of the liquid is again noted. The volume difference between the two marks is the hold-back volume.

About 70 ml. of the upper phase of the solvent mixture is added to the column reservoir and gas pressure is reapplied and pressure is adjusted so that the eluate flows from the column at about 1 ml./min. The eluate passes through a trap, the purpose of which is to prevent air bubbles and separated lower phase from entering the flow cell. A hypodermic syringe is used to withdraw lower phase from the trap if the level builds up too high. From the trap the eluate flows into a spectrophotometric flow cell attached to a Beckman model DU spectrophotometer set for determination of absorbance at 240 mμ. The resultant photo-current is recorded on a suitable recorder.

After passing through the detection device the eluate is collected in a series of graduated cylinders. According to the shape of the curve being recorded on the recorder various fractions are taken and the elution process is continued until no further ultraviolet-absorbing materials pass through the flow cell. Each component is characterized by a hold-back volume number, that is, the ratio of the total volume of eluate collected up to the midpoint of the peak as charted on the recorder to the hold-back volume as determined initially. Representative hold-back volume numbers (range of values) are tabulated in Table III for triamcinolone and some related steroids.

TABLE III.—REPRESENTATIVE NUMBERS OF HOLD-BACK VOLUMES OF TRIAMCINOLONE AND RELATED STEROIDS ON PARTITION COLUMNS

Steroid	Hold-Back Volumse in System: Dioxane/Cyclohexane/Water Ratio 5:2:1	5:3:1	5:4:1
Triamcinolone	3.2–3 7	4 3–7 2	ca. 10
1,2-Dihydrotriamcinolone	1 5–2.5	2.6–4.4	6
Triamcinolone isomer	4.7–5 2	8 1–12 0	ca. 12
1,2-Dihydrotriamcinolone isomer	3 0–3.5	7.2	9
9α-Fluorohydrocortisone	0.6–1.4	2.1	..
9α-Fluoroprednisolone	1.7	2.6	..

The several fractions taken according to the shape of the elution curve may be analyzed along any of the lines already described (1). For polarography the fraction is evaporated to dryness, care being taken to remove the last traces of solvent prior to dissolving in the polarographic solvent. Polarographic determination is then made in the usual manner (1). Colorimetric determination with tetrazolium blue is performed in the same way as previously described, care being exercised in the evaporation of the solvent to avoid heating. Each fraction from the column may be examined by paper chromatography for further evidence of its homogeneity and identity.

A process sample of triamcinolone analyzed with this method gave: triamcinolone, 95.0%; triamcinolone isomer, 2.1%; 1,2-dihydrotriamcinolone, 1.1%, with the total material balance (polarographic)

98.2%. The recovery of steroids polarographically in the eluted fractions averaged 96.8% in a series of 25 triamcinolone process samples, with a range of 89.7–102.4%.

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Enzymatic Decomposition of Digitalis Glycosides IV*

By OLE GISVOLD

IN A PREVIOUS PUBLICATION the glycosides found in the first year's growth of a number of species of digitalis have been reported (1). The glycosides were identified via paper chromatography using two very useful, dependable, quite temperature-insensitive solvent systems. In some cases the major glycoside was isolated in a crystalline state. Some of these plants survived the winter and bloomed in the summer of 1958. This permitted us to identify more accurately the individual species. It also gave me an opportunity to subject their leaves to the qualitative identification of their major glycosides. Extracts of the fresh leaves obtained by enzyme favoring and enzyme inhibiting techniques were examined by paper chromatographic techniques previously described (1). In all cases the same results were obtained as with the leaves from the first year's growth, i. e., the same desglucoglycosides were detected when enzyme favoring conditions were used and the same native glycosides when enzyme inhibiting techniques were employed. The species that were investigated were *Digitalis mertonensis*, *Digitalis amandiana*, *Digitalis siberica*, *Digitalis grandiflora*, and *Digitalis orientalis*. In the case of *Digitalis orientalis* the detectable desglucoglycosides were digoxin and acetyl digitoxin and in the case of *Digitalis grandiflora* they were digoxin, digitoxin, and acetyl digitoxin.

A previous investigation (2) showed that when *Digitalis purpurea* was dried rapidly below 60° larger amounts of the native glycosides could be detected. It was further demonstrated that these drying conditions did not inactivate the enzyme that removes the terminal glucose residue of the native glycosides. This discovery prompted a similar investigation of the dried leaves of the first year's growth of some of the other species of digitalis and analogous results were obtained when enzyme favoring and enzyme inhibiting techniques were employed. The same desglucoglycosides were detected when enzyme favoring conditions were used and the same native glycosides when enzyme inhibiting techniques were employed. The species investigated were *Digitalis mertonensis*, *Digitalis lanata*, *Digitalis orientalis*, and *Digitalis siberica*. It was also shown with the dried leaves of these species that 40 per cent methanol does not inhibit the enzyme that hydrolyzes the terminal glucose residue of the native glycosides. Furthermore, this alcohol concentration appeared to permit more complete hydrolysis as compared to the use of water or possibly 20 per cent methanol.

Although primary aqueous extracts of the fresh leaves of some of the digitalis species readily led to the isolation of some crystalline digitalis glycosides, the volume of water proved cumbersome, troublesome emulsions were encountered during extractions with organic solvents, and in some cases, there was incomplete extraction of the less water-soluble desglucoglycosides such as acetyl digitoxin and acetylgitoxin. The use of 15 per cent aqueous methanol was more effective than water and encouraged the use of greater concentrations of methanol or ethanol. Because 40 per cent concentrations of methanol did not inhibit enzymatic activity in the dried leaves of *Digitalis purpurea* whereas 66 per cent

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did, these concentrations were also used to extract the dried leaves of some of the other species of digitalis. In each case the results were analogous to those obtained with the dried leaves of *Digitalis purpurea*. The use of 40 per cent aqueous methanol permitted the ready extraction and subsequent detection of acetyl digitoxin in the case of *Digitalis lanata* whereas, when water was used to prepare the primary extract, the presence of acetyl digitoxin was difficult to establish.

The use of 40 per cent methanol to prepare the primary extracts had the following advantages: (*a*) more complete extraction of the less water-soluble desglucoglycosides, (*b*) much smaller volumes of solvent were needed, (*c*) easier filtration or percolation, and (*d*) less troublesome emulsions when the primary extract was extracted with organic solvents. The disadvantages that were encountered are: (*a*) incomplete extraction of the glycosides from the primary extract with organic solvents, (*b*) the necessity of diluting this primary extract with water or removal of part of the alcohol by distillation in order to obtain more complete extraction of the glycosides with organic solvents, and (*c*) considerably greater amounts of undesirable substances, some of which are colored, are obtained in this primary extract and in turn accompany the glycosides in the organic solvent extractions. The above discussion applied to both the qualitative identification and isolation studies.

In an attempt to improve upon the use of 40 per cent aqueous methanol, various concentrations of aqueous acetone or methyl ethyl ketone were tried. It was found that enzymatic activity in the dried leaves was not inhibited by 40 per cent aqueous acetone or 25 per cent aqueous methyl ethyl ketone. In these studies about the same advantages and disadvantages were encountered as with 40 per cent aqueous methanol. In the case of methyl ethyl ketone it was possible to add a sufficient amount of anhydrous sodium sulfate to effect a separation of the methyl ethyl ketone. A clean cut separation of the ketone was obtained and no emulsions were encountered. In this case the ketone served to facilitate the extraction of the glycosides in the primary extract and also served as the organic solvent to extract the glycosides from the primary extract. This methyl ethyl ketone extract contained considerable amounts of impurities in addition to the digitalis glycosides. Considerable amounts of impurities separated when a second solvent such as ether, isopropyl ether, etc., was added. The resultant mixed solvent solution of the glycosides was further partially depigmented by treatment with aqueous alkali as previously described (1).

Pigments, chiefly yellow in nature, accompany the organic solvent used to obtain the secondary extracts from the primary extracts. The quantities varied depending on the nature of the organic solvent or mixture of organic solvents. The solvents that have been tried are methyl isobutyl ketone, methylene dichloride plus ether (1:3), methyl ethyl ketone, and methyl ethyl ketone plus isopropyl ether (1:1). Considerable amounts of yellow pigment could be extracted from these secondary extracts with aqueous alkali and was most effective when the solvent was methylene dichloride ether (1:3). However, this procedure did not remove all the yellow pigments some of which were troublesome during qualitative paper chromatographic analyses. After removal of the organic solvent from the partially depigmented secondary extracts the residue was dissolved in methanol. In this solvent, especially if small amounts of water are added, more pigments can be removed with the base form of the ion exchange resin IRA-401.

Powdered dried leaves of *Digitalis mertonensis* were used in order to prepare larger quantities of digitoxin using 40 per cent methanol to prepare a primary extract. Contrary to previously reported findings (1), I was not able to obtain digitoxin as readily in a crystalline state, even though the final preparation contained a high percentage of digitalis glycosides. Analysis by paper chromatography, before and after deacetylation, revealed that this preparation contained considerable quantities of acetyl digitoxin together with larger amounts of digitoxin. These results were substantiated when the above experiment was repeated several times, on both small or large scales using dried leaves, and by a variety of extraction techniques. This was quite surprising since earlier techniques had led to the ready isolation of crystalline digitoxin when fresh leaves were employed and water or 15 per cent aqueous methanol was used as the solvent to prepare the primary extract. One might conclude that in the latter case the temperatures employed or enzymes in the fresh leaves led to a large amount of deacetylation of the acetyl digitoxin. If this be the case, it would differ from the results obtained with *Digitalis siberica* where only acetyl digitoxin could be detected even though, in this case, the primary extract was made with water or 15 per cent aqueous methanol in the same way. At the present stage of my investigations and for the lack of fresh leaves, no clear-cut conclusion can be drawn as to whether or not both purpurea glycoside A and lanatoside

A are present as native glycosides of *Digitalis mertonensis*. Further studies on fresh leaves should clarify this situation.

It should be pointed out at this time that *Digitalis mertonensis* is a hybrid of the *Digitalis purpurea* and *Digitalis grandiflora* species. The former yields purpurea glycoside A which, upon partial hydrolysis, leads to digitoxin whereas in the latter case lanatoside A is produced which can lead to acetyl digitoxin.

EXPERIMENTAL

The details of the paper chromatographic techniques used in these studies have been described previously (1–3) Both solvent systems I and II were used for the development of the paper chromatograms The Raymond reagent or Jensen's trichloroacetic acid reagent (4) were used to detect the position of the glycosides on the paper. The extracts also were prepared as previously described (1, 2), *unless otherwise specified.*

Extraction With Methyl Ethyl Ketone. Dried powdered *Digitalis siberica*, 200 Gm., was macerated with 1,000 cc. of 25% aqueous methyl ethyl ketone for eighteen hours. It was then packed in a percolator and slowly percolated with the same menstruum, and 1,500 cc. of percolate was collected. A sufficient amount of anhydrous sodium sulfate was added to effect a separation of the ketone (75% of the original amount of the ketone separated). The aqueous layer was extracted with a second quantity, 200 cc. of methyl ethyl ketone. The ketone extracts were combined and the solvent removed under vacuum. The residue was diluted with two heaping teaspoons of Celite filter aid and subsequently extracted with isopropyl ether—Skelly B, 1:1 (by volume) to remove some lipids and some pigments. It was then extracted with 100 cc. of methylene dichloride which was concentrated to a volume of 20 cc. and diluted with 60 cc. of ether. This extract was almost decolorized by extracting it with two 10-cc. portions of 5% aqueous potassium hydroxide. The organic solvents were removed and acetyl digitoxin crystallized from the residue using a mixture of methylene dichloride and isopropyl ether. The crystallization of acetyl digitoxin was very slow and required a considerable amount of time even when seeded.

A second method of utilization of methyl ethyl ketone was as follows: 10 Gm. of dried, powdered *Digitalis mertonensis* was heated at 60° with 150 cc. of water for one hour. The mixture was filtered and the drug washed with 50 cc. of hot (80°) water. The filtrate, 160 cc., was diluted with 40 cc. of methyl ethyl ketone. Anhydrous sodium sulfate then was added to effect a separation of the ketone. No emulsion was encountered. The aqueous layer was extracted with a second portion (30 cc.) of methyl ethyl ketone. The ketone extracts were combined and treated as described above. In addition, a methanolic solution (4 cc.) of the final product was treated with the powdered base form of IRA-401 which removed considerable additional amounts of yellow pigments. The final preparation, when examined paper chromatographically, revealed the presence of digitoxin and acetyl digitoxin. The identity of acetyl digitoxin was further established when, after deacetylation either by IRA-401 (base form) in hot 85% aqueous methanol or 0.1 *N* NaOH in methanol, paper chromatographic analyses showed the disappearance of acetyl digitoxin with an increase in the intensity of the digitoxin spot and the appearance of no new glycoside.

This experiment was designed to favor deacetylation *because water and temperatures under 60°* appear to favor deacetylation in the case of fresh leaves In another experiment the primary aqueous methyl ethyl ketone extract was boiled for one hour; however, acetyl digitoxin could be readily detected. At the present stage of my researches the status of digitoxin and acetyl digitoxin as desglucoglycosides from *Digitalis mertonensis* needs further investigation.

Extraction With 40% Aqueous Methanol.—Dried, powdered *Digitalis mertonensis*, 10 Gm., was digested for five days with 100 cc. of 40% aqueous methanol. After filtration the marc was washed with 50 cc. of the same solvent. This primary extract was concentrated to remove most of the alcohol. It was then extracted with methyl isobutyl ketone and further purified in the usual way. Paper chromatographic examination showed the presence of digitoxin and acetyl digitoxin, and the latter seemed to predominate. This experiment was performed to see (*a*) if deacetylation would take place over this period of time and (*b*) to see if the glycosides would remain intact. Shorter periods of maceration gave similar results.

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(4) Svendsen, A. D., and Jensen, K. B., *Pharm. Acta He*[illegible] . K. B., *Acta Pharmacol. Toxicol.*, 9, [illegible] and Thorp, R. H., *J. Pharm. an*[illegible]

Reactivity of Bisulfite With a Number of Pharmaceuticals*

By TAKERU HIGUCHI and LOUIS C. SCHROETER

Although bisulfite is presently widely employed as an antioxidant in preparations containing drugs which readily autoxidize; the antioxidant itself, according to the study described, undergoes reaction with a number of these compounds. Results of the present investigation show that sympathomimetic and other drugs which are *para-* or *ortho*-hydroxybenzyl alcohol derivatives react with bisulfite to yield corresponding sulfonic acid derivatives. Both amino and nitrobenzyl alcohols similarly conjugated (e. g., chloramphenicol) also appear to be subject to similar reactions. The resulting sulfonic acids seem to possess little or no activity.

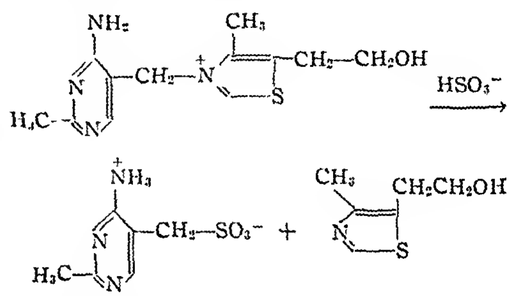
Bisulfite has been employed in pharmaceutical preparations as a stabilizer for many years. It has, in many cases, served very well in this capacity and inclusion of bisulfite into preparations as an antioxidant has become an accepted procedure. Attainment of a clear solution or elegant preparation through the use of bisulfite with easily oxidizable compounds has done much to support its use in formulations. In the present communication it is shown, however, that the stability of many important pharmaceuticals may be seriously impaired by the presence of bisulfite.

The reaction of aldehydes or certain ketones with bisulfite and the addition of bisulfite to the alkene linkage has been intensively studied (1, 2) and reactivity of these compounds is generally recognized. Reaction of alkyl halides with alkaline sulfite in aqueous solution to yield alkyl sulfonic acids is somewhat less known. Clutterbuck and Cohen (3) report the formation of a number of substituted benzyl sulfonic acids by boiling the corresponding benzyl bromide with aqueous sodium sulfite. The mechanism for this reaction appears to involve nucleophilic attack of sulfite at the partially positive carbon to yield a carbon-sulfur bond:

$$R{-}\overset{\delta^+}{CH_2}{-}\overset{\delta^-}{X} + SO_3^{=} \longrightarrow R{-}CH_2{-}SO_3^- + X^-$$

The interaction of aromatic nitro compounds with dilute sulfite solution to yield an arylaminosulfonic (arylsulfamic) acid and its sulfonation product takes place under similar conditions and is known as the Piria reaction (4). Nuclear sulfonation takes place *ortho* or *para* to the nitro group, presumably due to a nucleophilic attack at the partially positive carbon atom.

Early attempts to preserve rice polish extracts with sulfurous acid led to prompt and complete loss of thiamin activity. Further studies (5) showed that thiamin was cleaved by bisulfite into two compounds, one of which was a sulfonic acid. Thiamin cleavage of bisulfite has been demonstrated (6, 7) to occur as follows:



Experiments (6) with tritium indicated hydrogen is not exchanged in or out of the interannular methylene bridge of thiamin or of the sulfonic acid during the cleavage process. It appears that the pyrimidine sulfonic acid results from a nucleophilic attack on the partially positive methylene carbon.

In view of these observed reactions involving bisulfite, it is evident that the use of the antioxidant may result in instances of reduced stability. An example of this is the recently reported degradative route of epinephrine (8). The present study is concerned with this reaction and with determination of the types of drugs which may exhibit tendencies in this direction. Specifically, results of investigations on the reaction of bisulfite with epinephrine, ephedrine, 2-amino-1-phenylethanol, 2-methylamino-1-phenylethanol, catechol, resorcinol, quinhydrone, Methadren, N-acetylepinephrine, Synephrine, Neosynephrine, *p*-hydroxytoluene, *p*-methoxybenzyl alcohol, methyl-*p*-hydroxybenzoate, salicylamide, salicyl alcohol, *m*-hydroxybenzyl alcohol, *p*-hydroxybenzyl alcohol, *p*-aminobenzyl alcohol, *p*-aminobenzoic acid, 4-aminobenzoic acid, 4-aminosalicylic acid, tartaric acid, starch, chloramphenicol, and *p*-nitrobenzyl alcohol are reported.

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EXPERIMENTAL

Epinephrine solutions were prepared in 0.1 *M* acetate or phosphate buffers. The pH was determined at 25° using a Beckman model G pH meter. The solutions were flushed with nitrogen and stored under a positive nitrogen atmosphere until ready for use. These solutions served as blanks. Identical solutions were prepared containing, in addition, varying concentrations of sodium bisulfite or sodium sulfite. The pH of these solutions was adjusted at 25° to that of the appropriate blank and then flushed with nitrogen. Solutions were filled into a jacketed-polarimeter tube designed so that a nitrogen atmosphere was maintained above the solution at all times. Polarimetric measurements were made with a Zeiss-Winkel polarimeter using 589 mμ filtered sodium light at the temperature of the thermostat.

Bisulfite or sulfite solutions were prepared in 0 1 *M* acetate or phosphate buffers. The pH was determined at 25°. These blank solutions were flushed with nitrogen then rapidly filled into hard glass ampuls Identical solutions were prepared containing, in addition, varying concentrations of epinephrine. The pH was adjusted at 25° to that of the appropriate blank, then flushed with nitrogen and rapidly filled into hard glass ampuls. The filled ampuls were flushed with nitrogen, evacuated, and sealed under vacuum. The sealed ampuls were stored at a constant thermostatically controlled temperature which varied less than 0.1°. Sampling was performed by periodically removing ampuls and quickly chilling in an ice-water mixture. Prior to analysis, ampuls were adjusted to 25°. The pH of all solutions subjected to elevated temperature was determined. Available bisulfite was determined by iodometric titration.

Reactivity of bisulfite with the following compounds was tested as described above: Ephedrine, U. S. P ; 2-amino-1-phenylethanol and 2-methylamino-1-phenylethanol synthesized from styrene bromohydrin according to the method of Read and Reid (9); catechol; resorcinol; quinhydrone; Methadren (N-methylepinephrine)[1]; N-acetylepinephrine, m. p. 135° obtained by hydrolysis (pH 7.5 phosphate buffer) of O[3], O[4], N-triacetylepinephrine; Synephrine and Neosynephrine[2]; *p*-hydroxytoluene; *p*-methoxy-benzyl alcohol; methyl-*p*-hydroxybenzoate; salicylamide; salicyl alcohol; *m*-hydroxybenzyl alcohol; *p*-hydroxybenzyl alcohol; *p*-aminobenzyl alcohol; *p*-aminobenzoic acid; 4-aminosalicylic acid; tartaric acid; and starch U. S. P.

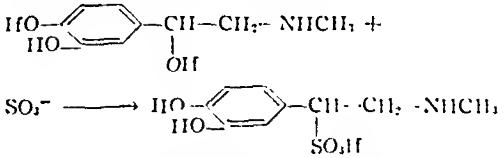
The reaction product resulting from bisulfite with *ortho*-hydroxybenzyl alcohol was prepared in quantity by heating in aqueous solution of 0.1 *M* salicyl alcohol and 0.2 *M* sodium sulfite in an evacuated, sealed ampul at 80° for periods up to one hundred hours. After cooling, the solution was extracted four times with equal volumes of ether An aliquot was oxidized by alkaline permanganate and compared with *ortho*-sulfobenzoic acid obtained by acid hydrolysis of *saccharin*. The aqueous solution was made distinctly acid with hydrochloric acid (pH 2) and the solution again extracted with ether. An aliquot of the aqueous acid solution was boiled with 2,4-dinitrophenylhydrazine solution. Part of the aqueous acid solution was basified with sodium hydroxide and methylated under alkaline conditions with dimethylsulfate. The basic solution was extracted four times with equal volumes of ether. The methylated product was compared with known *ortho*-methoxybenzyl sulfonic acid synthesized by the method of Clutterbuck and Cohen (3) from the corresponding benzyl bromide and sodium sulfite solution. Both products were reacted with phosphorus pentachloride (0.5 of sod. sulfonate + 2 Gm. PCl_5) at 150° under reflux for forty minutes. Residue was extracted with benzene. Benzene solutions were evaporated to dryness. The residue was boiled with concentrated ammonium hydroxide solution.

Chloramphenicol and *p*-nitrobenzyl alcohol solutions were prepared in 0.1 *M* phosphate buffers and flushed with nitrogen prior to filling into ampuls. Chloramphenicol solutions were also prepared with L-threo-chloramphenicol in ethylene glycol-water (80:20). These solutions served as blanks. Identical solutions were prepared containing, in addition, varying concentrations of sodium bisulfite or sulfite. Ampuls were sealed under vacuum and stored for varying periods in a thermostat. Bisulfite loss from solutions was followed by iodometric titration. Chloramphenicol was assayed by a chromatographic procedure (10) and the optical activity (ethylene glycol-water solutions) determined at 25° in a 2-dm. micro polarimeter tube using 589 mμ light.

RESULTS AND DISCUSSION

Reaction with Epinephrine

Studies with epinephrine and optically active model compounds indicate that loss in optical activity occurs simultaneous to epinephrine and bisulfite loss. Evidence strongly suggests the asymmetric secondary alcohol carbon of epinephrine as the reactive site in this reaction:



The course of the reaction seems to be somewhat dependent on the pH of the reacting system.

At pH of 6 5, the reaction appears to be definitely a second-order reaction where $-d(\mathrm{Ep})/dt = -d(\alpha)/dt = k\,(\mathrm{Ep})\,(\mathrm{Bi})$ as shown in Table I. The bisulfite concentration being in this instance the total of sulfite and bisulfite concentration. This relationship is also evident in the logarithmic type plot shown in Fig. 1 for systems starting with dissimilar concentrations as followed by bisulfite titration.

Loss of optical activity appears to occur at the same rate as loss of bisulfite and equilibrium optical activity of solutions is always zero. The following scheme will readily explain zero optical activity:

$$\mathrm{Ep}(-) \xrightarrow[\text{(slow)}]{} \mathrm{Ep}(\pm) \xrightarrow[\text{(fast)}]{(SO_3^-)} \mathrm{EpBi}(\pm)$$

(± refers to sign of optical rotation)

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[1] Samples generously supplied by Lakeside Laboratories, Milwaukee, Wis

[2] Samples generously supplied by Sterling-Winthrop Research Institute, Rensselaer, N. Y.

TABLE I.—INITIAL RATES OF BISULFITE AND OPTICAL ACTIVITY LOSS FROM SOLUTIONS BUFFERED AT pH 6.5 WITH 0.1 *M* PHOSPHATE[a]

Temp., °C.	Soln. A[b] $-d\alpha/dt$ (mole l.$^{-1}$ sec.$^{-1}$)	Soln. B[c] $-d(\text{Bi})/dt$ (mole l.$^{-1}$ sec.$^{-1}$)
90	6.63×10^{-6}	6.73×10^{-6}
84	3.65×10^{-6}	3.58×10^{-6}
78	2.09×10^{-6}	1.96×10^{-6}
72	1.21×10^{-6}	1.09×10^{-6}
66	7.12×10^{-7}	5.96×10^{-7}

[a] Initial rate of loss for reactant present in lesser concentration where concentration change is less than 10%.
[b] Solution A—2.75×10^{-2} *M* epinephrine, 9.60×10^{-2} *M* sodium bisulfite. Optical activity assumed to be due to *levo*-epinephrine.
[c] Solution B—1.92×10^{-2} *M* sodium bisulfite, 5.50×10^{-2} *M* epinephrine.

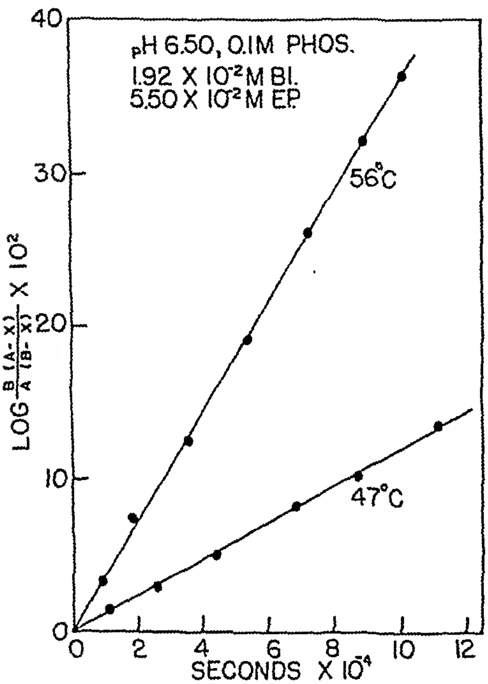


Fig. 1.—Typical plots establishing second-order characteristics of epinephrine (Ep)-sodium bisulfite (Bi) reaction at pH 6.50 and various temperatures. A—5.50×10^{-2} *M* epinephrine base (Ep) and B—1.92×10^{-2} sodium bisulfite (Bi). Rate constants were calculated from slopes: $k = m \times 2.303/A - B$.

This is the form of the S_{N2} mechanism. Implied in this mechanism is dependence of reaction only on epinephrine. If this were the only mechanism, the rate of bisulfite loss would equal the rate of loss of optical activity; however, observed second-order kinetics at higher pH values suggest additional pathway(s). Prior studies (8) indicate that this mechanism may make significant contributions to the reaction at pH values below 4.5.

At higher pH values, a slow, rate-determining S_{N2} reaction between epinephrine and bisulfite followed by rapid racemization seems a distinct possibility. In this case, one must assume rapid racemization since the S_{N2} mechanism generally involves inversion of configuration at the optical center and this would tend to create a positive equilibrium optical rotation. Kinetic evidence indicating that the reaction is second order at pH 6.5 suggests the following:

$$\text{Ep}(-) + \text{Bi} \xrightarrow[\text{slow}]{} \text{EpBi}(+) \underset{(\text{fast})}{\rightleftarrows} \text{EpBi}(-)$$

Isolation of bisulfite-salicyl alcohol as discussed below strongly suggests the formation of a benzyl sulfonic acid and, if the reaction in this model compound is similar to that of epinephrine, one would expect that the epinephrine-bisulfite product is also a benzyl sulfonic acid.

Bisulfite Reactivities of Other Compounds

Results of a screening test with a number of pharmaceuticals having close or distant similarity to epinephrine are given in Table II. In all cases a substantial excess of the drug was used and the bisulfite concentration followed by iodometric titration. The rate is expressed in terms of pseudo half-life at the given concentrations of the medicinal agents.

Ephedrine and Analogs.—Variation of reactant molarity, pH, or buffer failed to give a detectable reaction between ephedrine and bisulfite. Structural differences between ephedrine and epinephrine suggested that either the phenolic hydroxyls of epinephrine or the slightly different side chain accounted for the lack of reactivity of ephedrine with bisulfite. Failure of both 2-amino-1-phenylethanol and 2-methylamino-1-phenylethanol to react with bisulfite indicated side chain involvement to be a questionable hypothesis.

Dihydroxy Benzenes.—The isomeric dihydroxybenzenes (catechol, resorcinol, and the hydroquinone-quinone combination) also failed to react with bisulfite under standard conditions of the study. Reaction between bisulfite and hydroquinone and resorcinol on prolonged heating has been reported (11) but no loss of bisulfite was detected in our study. This seemed to indicate that the overall reaction between epinephrine and bisulfite involved the phenolic function with at least part of the side chain.

Epinephrine Analogs.—Neosynephrine, differing from epinephrine only in lacking the 4-hydroxyl, failed to react with bisulfite; however, Synephrine (*p*-methylaminoethanol phenol) reacted rapidly with bisulfite. Methadren (N-methylepinephrine) likewise underwent rapid reaction with bisulfite further indicating that the *p*-hydroxybenzyl alcohol portion of the molecule was involved in the reaction. Under identical reaction conditions, the pseudo first-order rate constant for the Methadren-bisulfite reaction was found to be approximately the same as that for the epinephrine-bisulfite reaction. Basicity difference between the secondary and tertiary amine apparently does not much influence the reaction rate. The N-acetylepinephrine reaction with bisulfite occurs at a slower rate than that found with epinephrine or Synephrine. The protonated amine in epinephrine, pKa 8.55, and Synephrine, pKa 8.62 (12), appears to facilitate the reaction.

Catechol-Phenylephrine Combination.—Evidence against sequential reaction of bisulfite with side chain, followed by attack on phenolic hydroxyls

Table II.—Reactivity of Various Compounds at 68° with 1.92 × 10^{-2} M Sodium Bisulfite[a]

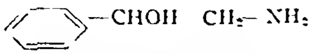
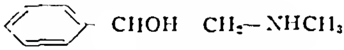
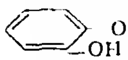
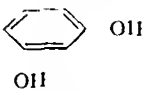
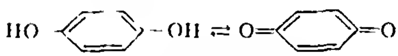
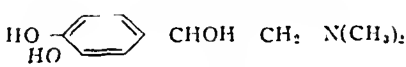
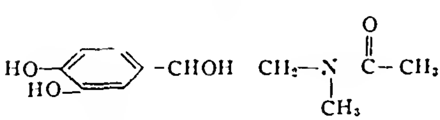
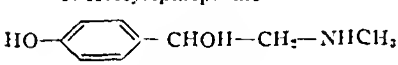
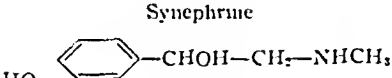
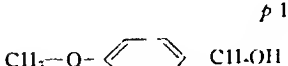
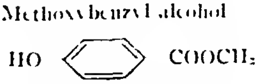
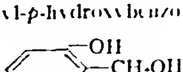
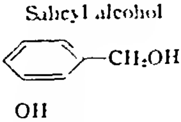
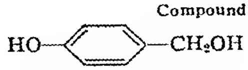
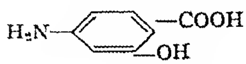
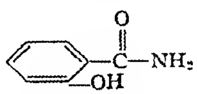
Compound	Concn.	Buffer pH[b]	Bisulfite Half Life, hr.	Total Residence Time, hr.
 Ephedrine	0.023 M	5.50, Ac	N.R.	76
	0.115 M	5.50, Ac	N.R.	76
	0.023 M	8.50, Phos	N.R.	67
 2-amino-*l*-phenyl-ethanol	0.073 M	5.50, Phos	N.R.	144
 2-Methylamino-*l*-phenylethanol	0.033 M	5.00, Ac	N.R.	117
 Catechol	0.090 M	4.50, Ac	N.R.	168
	0.090 M	6.70, Phos	N.R.	113
 Resorcinol	0.090 M	6.80, Phos	N.R.	111
 Quinhydrone	0.090 M	6.50, Phos	N.R.	96
 Methadren	0.043 M	5.50, Phos	30	
	0.026 M	5.50, Ac	12	
 N-Acetylepinephrine	0.016 M	6.50, Phos	83	
 Synephrine	0.050 M	6.40, Phos	32	
	0.055 M	6.98, Phos	10	
 Neosynephrine	0.050 M	5.50, Phos	N.R.	111
	0.050 M	6.70, Phos	N.R.	111
Catechol Phenylephrine	0.045 M			
	0.025 M	5.50, Ac	N.R.	110
 *p*-Hydroxytoluene	0.092 M	6.50, Phos	N.R.	168
 *p*-Methoxybenzyl alcohol	0.040 M	8.00, Phos	N.R.	96
 Methyl-*p*-hydroxybenzoate	0.033 M	7.00, Phos	N.R.	120
 Salicyl alcohol	0.080 M	6.50, Phos	10	
 *m*-Hydroxybenzyl alcohol	0.040 M	6.70, Phos	N.R.	18
	0.080 M	6.70, Phos	N.R.	120

TABLE II—*Continued*

Compound	Concn.	Buffer pH[b]	Bisulfite Half-Life, hr.	Total Residence Time, hr.
 *p*-Hydroxybenzyl alcohol	0 040 *M*	6.50, Phos.	9	.
 *p*-Aminobenzyl alcohol	0 080 *M*	7 20, Phos.	11	..
 *p*-Aminobenzoic acid	0 036 *M*	6 80, Phos.	N.R.	96
 4-Aminosalicylic acid	0 033 *M*	6 80, Phos.	N.R.	96
 Salicylamide	0 036 *M*	7.00, Phos.	N.R.	120
HOOC—CHOH—CHOH—COOH Tartaric acid	0 067 *M*	7 20, Phos.	N.R.	120
Starch U. S. P.	0 5%	7 20, Phos.	N.R.	120

[a] Solutions stored in evacuated, sealed ampuls
[b] 0 1 *M* acetate (Ac) or 0 1 *M* phosphate (Phos) buffers.

or vice versa was provided by the study of the catecol-phenylephrine combination with bisulfite. Failure of the combination to react with bisulfite further substantiates the idea of the *p*-hydroxybenzyl alcohol moiety of epinephrine as the active portion.

***p*-Hydroxytoluene, *p*-Methoxybenzyl Alcohol, and Methyl-*p*-hydroxybenzoate.**—The simple model compounds, *p*-hydroxytoluene, *p*-methoxybenzyl alcohol, and methyl-*p*-hydroxybenzoate, show no reaction with bisulfite under standard conditions. These compounds are all *para*-substituted benzenes; however, resonance contributions to give the quinoid structure are not nearly so large as in the *para*-hydroxybenzyl alcohol.

Hydroxybenzyl and *p*-Aminobenzyl Alcohols.—The isomeric hydroxybenzyl alcohols show very significant information in that the *ortho* and *para* isomers react rapidly with bisulfite while the *meta* does not. This confirms the results obtained with Synephrine and Neosynephrine which are *para*- and *meta*-hydroxybenzyl alcohol derivatives, respectively. Involvement of contributing resonance forms seems apparent at this point and studies with *p*-aminobenzyl alcohol strongly suggest the *para*-quinoid structure of a benzyl alcohol as the reactive form Further, the reaction of *p*-amino-benzyl alcohol obviates consideration of the phenolic group *per se*.

***p*-Aminobenzoic Acid, 4-Aminosalicylic Acid, and Salicylamide.**—Studies with *p*-aminobenzoic acid, 4-amino salicylic acid, and salicylamide indicate that the aromatic amino group and the phenolic hydroxyl group do not account for the bisulfite reaction in themselves These groups, when *para* or *ortho* to the benzyl alcohol, make the quinoid resonance form a distinct possibility and activate the alcohol.

Tartaric Acid and Starch U. S. P.—As expected, no reaction was detected with the common formulation ingredients, tartaric acid, and starch U. S. P.

Salicyl Alcohol–Bisulfite Product.—Reaction of salicyl alcohol with sodium bisulfite in a sealed tube yields a polar compound insoluble in ether and showing the same ultraviolet spectrum as salicyl alcohol. Combustion analysis of the product indicated the formula: $C_7H_7O_4NaS$. Alkaline permanganate oxidation of the product yields no isolable or ultraviolet-detectable aromatic products. If the reaction involved substitution at the phenolic hydroxyl group, oxidation should have yielded *ortho* sulfobenzoic acid.

Acidification of the salicyl alcohol-bisulfite product failed to yield an ether-soluble product and boiling the acid solution with 2,4-dinitrophenylhydrazine failed to yield a derivative. An aldehyde intermediate in the reaction seems unlikely. Methylation of the salicyl alcohol-bisulfite product with dimethylsulfate under basic conditions produced a polar compound insoluble in ether which showed a doublet peak in the ultraviolet. The doublet peak at 273 and 280 mμ was identical with the *ortho*-methoxybenzyl chromophore.

The methylated product and known *ortho*-methoxybenzyl sulfonic acid were subjected to alkaline permanganate oxidation under a reflux condenser for periods up to six hours. Less than 10% *ortho*-methoxybenzoic acid was isolated from the mixture. The remainder of the product in each case is unchanged indicating the inordinate resistance of benzyl sulfonic acids to alkaline hydrolysis and alkaline oxidation.

Both the methylated product and known *ortho*-methoxybenzyl sulfonic acid failed to yield sulfuryl chloride derivatives by usual phosphorus pentachloride procedure. Reaction residues contained varying amounts of *ortho*-methoxybenzyl chloride.

Formation of benzyl chlorides rather than expected sulfonyl chlorides has been previously reported (1). Apparently strict control of reaction conditions is necessary to obtain benzyl sulfonamides since Clutterbuck and Cohen (3) report preparations of sulfonamides derivatives for substituted benzyl sulfonic acids other than *ortho-* and *para*-methoxybenzyl sulfonic acid

Chloramphenicol.—Degradation of chloramphenicol induced by bisulfite appears to be considerably more complex than that found with epinephrine. Certain similarities may be seen in Fig. 2 in which both the log of chloramphenicol loss as determined by chromatography assay and log of optical activity are plotted against time. The reaction follows pseudo first-order kinetics since the bisulfite is initially in excess. Loss in optical activity occurs at a rate much greater than that observed in the absence of bisulfite. Presence of two optically active carbon atoms imposes an element of uncertainty in evaluating polarimetric data and, to be sure, the initially levorotatory solutions were found to yield dextrorotatory solutions at equilibrium (15 half-lives) Further, the stoichiometry of

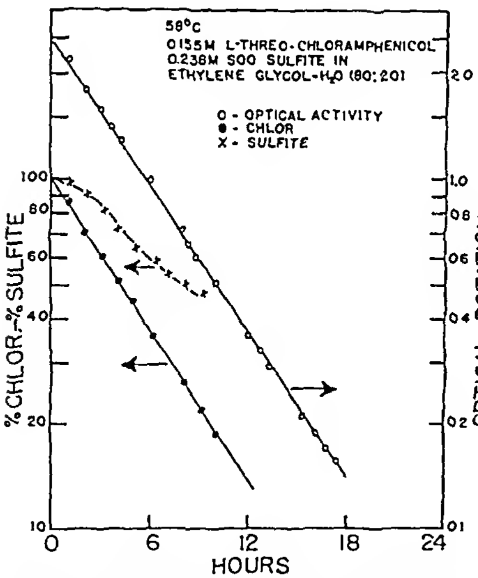


Fig. 2.—Ethylene glycol-water (80:20) solution of 0.155 *M* L-threo-chloramphenicol and 0.238 *M* sodium sulfite at 58°. Per cent chloramphenicol (Chlor.) and per cent sulfite plotted logarithmically on left axis; optical rotation in degrees measured at 25° in 2-dm. polarimeter tube plotted logarithmically on right axis.

the reaction is complicated by the reductive tendency of bisulfite with nitro compounds and its nucleophilic reactivity toward alkyl halides.

Studies with the model compound, *p*-nitrobenzyl alcohol, indicated very rapid loss of bisulfite from solutions with concomitant appearance of a polar compound. Production of the polar product was followed by partition studies of the solutions. As with chloramphenicol, the initial rate loss of the nitro compound is near that of bisulfite; however, as the reaction proceeds, the bisulfite loss tends to increase, indicative of side reactions.

GENERAL OBSERVATIONS

Comparative study of epinephrine and ephedrine analogs and model compounds clearly indicated that the reaction between epinephrine and bisulfite is not unique. The basis for the reactivity of epinephrine or similar drugs with bisulfite under conditions of the experiment has been shown to involve the hydroxybenzyl alcohol portion of the molecule.

The influence of position in the reactivity of the isomeric hydroxybenzyl alcohols indicated that resonance contributions to a quinoid structure would account for lack or reactivity of *meta* isomer. Correctness of this concept was substantiated in studies with *p*-aminobenzyl alcohol, which has considerable quinoid resonance contribution.

The chloramphenicol study showed certain similarities to the epinephrine reaction in that optical activity loss occurs at nearly the same rate as the loss of chloramphenicol. The reaction appears to be more complex than that observed with hydroxybenzyl alcohol derivatives. Studies with the model compound *p*-nitrobenzyl alcohol indicated strong possibility for nitro reduction.

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Protective Effects of Air Under Pressure on Certain Pharmaceuticals During Steam Autoclaving*

By LEON LACHMAN, DONALD JACONIA, and PHILIP EISMAN

Pharmaceutical products such as jellies packaged in collapsible tubes cannot be properly sterilized in conventional steam autoclaves because of expansion or bursting of the tubes. Similarly, leakage at the cap occurs during the sterilization of screw-capped bottles. In this report, a redesigned steam autoclave is described which permits the practical sterilization of these pharmaceutical formulations. This autoclave is a dual-control unit permitting the introduction of air under pressure into the chamber so as to obtain a total air plus steam pressure greater than that of the vapor pressure within the containers. In addition, this unit allows for a reduction in chamber temperature without loss of chamber pressure during the cooling cycle. The suitability of this dual-control autoclave for the sterilization of the aforementioned products under various conditions of temperature and pressure was determined. Data are presented indicating that these pharmaceutical products can be effectively sterilized without deleterious effects to the containers. The jellies and solutions used in this investigation were heavily contaminated with *B. cereus* spores. Recordings of the temperature within the containers and chamber show that there is a lag in the rise and fall of the temperature within the containers as compared to that of the chamber.

Although there are a number of techniques involving heat which may be employed for the sterilization of various pharmaceutical dosage forms, there is little doubt that the most dependable procedure is the one which utilizes steam under pressure. Unless the product to be sterilized is deleteriously affected by the temperature and pressure involved, the autoclave serves admirably in destroying all forms of microbial life

The effectiveness of this method of sterilization is dependent upon two essential factors, namely moisture and heat It is a universally accepted fact that the thermal destruction of microorganisms parallels the principles involved in the heat coagulation of proteins In the presence of adequate amounts of water, the coagulation of proteins occurs at temperatures well below that necessary to achieve the same results when the moisture content is appreciably reduced This relationship between coagulation temperature of proteins and moisture content was presented in a classic paper by Lewith (1) in 1890, a summary of which is shown in Table I

TABLE I.—COAGULATION OF EGG ALBUMIN WITH VARYING MOISTURE CONTENT

Egg Albumin-Water Mixtures	Temperature of Coagulation in 30 Min., °C
Aqueous solution	56
With 25% water	74–80
With 18% water	80–90
With 6% water	145
Water free	160–175

The term autoclave, as it is usually applied, refers to a chamber capable of accepting pressurized steam under controlled conditions The sterilizing power of the steam is a function of its temperature and not its pressure. However, since the temperature in the autoclave is proportional to its steam pressure, an increase in pressure results in an increase in temperature

Not all pharmaceutical products can be suitably sterilized in an autoclave In the process of autoclaving, pressure is built up within the container being sterilized because of the vapor pressure of water and the partial pressures of volatile components, if present in the formulation. Under these circumstances, the internal pressure could be substantially greater than the steam pressure within the chamber itself. This pressure differential could result in collapsible tubes expanding and bursting at the crimp end, screw-capped bottles losing a portion of their contents, and the breaking of glass containers Furthermore, during the cooling phase of the sterilization process, the chamber almost always cools at a faster rate than does the contents of the sterilized packages. As a consequence, pressures within these containers are substantially higher than that of the chamber, and unless these containers are properly sealed and adequately constructed, deleterious effects may result. Containers such as collapsible tubes, whether constructed of aluminum or plastic, bottle assemblies fitted with screw caps, and Fenwall bottles with their not-too-firmly anchored caps are particularly subject

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The authors wish to acknowledge their appreciation to Mr Jack Cooper and Mr Jack Lazarus whose initial development work in the terminal sterilization of collapsible aluminum tubes in modified experimental and production steam pressure autoclaves led to the concept of the dual pressure autoclave We are also indebted to Mr William Glen Mr Robert McDonald, and Mr H G Boucher of the Wilmot Castle Laboratories for designing the dual control autoclave used in this study.

to this undesirable effect of uneven pressures For this reason, such products as sterile jellies and ophthalmic solutions are often commercially prepared according to aseptic techniques.

This difficulty, namely the bursting of collapsible tubes and the leakage of solution from screw-capped containers, can be overcome by the simple expediency of controlling chamber pressure through the introduction into the chamber of air under pressure during the heating and subsequent cooling cycles However, the addition of air to steam results in a mixture which, at any particular total pressure, has a temperature lower than that obtained with saturated steam alone at the same pressure (2). This relationship of air plus steam pressure to temperature is exemplified by the data presented in Table II. Thus, if one operates an autoclave in which only one-half of the air in the chamber has been removed, a pressure of 15 p. s. i. results in a temperature of 112° as contrasted to a temperature of 121° in the case where all the air has been evacuated. As illustrated in Fig. 1, one can predetermine the temperature resulting from the use of any proportion of air to steam by plotting the chamber temperatures against different proportions of air and steam at a constant total pressure.

Consideration was then given to the possibilities of utilizing air-steam mixtures in the sterilization of products not normally amenable to conventional autoclaving treatment because of leakage or other undesirable physical effects to the containers. Excellent examples of such products are pharmaceutical jellies packaged in collapsible aluminum tubes and ophthalmic solutions in screw-capped bottles. Through the cooperation of the Wilmot Castle Company Laboratories in Rochester, N Y., an autoclave was redesigned so as to permit the regulation of chamber temperature and pressure independently of each other. This redesigned autoclave was made available to us for use in this study and hereafter will be referred to as the "dual-control autoclave."

The objects of this study were (*a*) to determine the sterilizing effectiveness of contaminated pharmaceutical jellies and ophthalmic solutions subjected to various combinations of temperature and pressure in the dual-control autoclave; and (*b*) to determine the ability of the tubes and bottles to withstand physical changes under these conditions In addition, simultaneous temperature recordings were made of the autoclave chamber and the contents of the containers in order to determine whether a substantial time lag occurs between the rise and fall of the temperature within the tubes and bottles in relation to the chamber temperature

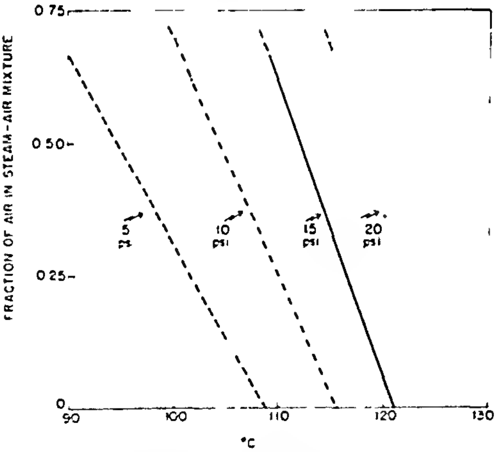

Fig 1 A plot of air-stream mixtures *vs* temperature at different total gauge pressure

EXPERIMENTAL

Design of the Dual-Control Autoclave

As shown in Fig 2, this unit is essentially an adapted steam autoclave of the ordinary type in which it is possible (*a*) to build up pressure without heat, (*b*) to utilize temperatures below 100° without pressure, and (*c*) to apply varying temperatures and pressures concurrently. These features are brought about by the introduction of solenoid-controlled air and steam at the inlet port of the sterilizer The steam serves to heat the chamber and when the desired temperature is reached, air is introduced to bring the chamber to the required pressure For example, in order to operate the autoclave at 105° at 15 p s i, steam is passed into the chamber until a temperature of 105° is reached Since at this temperature there is a pressure of 3 p s i, air is admitted until the total pressure of 15 p s. i is obtained The thermo and pressure switches on the chamber automatically maintain the desired temperature and pressure during the sterilizing cycle A pulsator system

TABLE II.—STERILIZER TEMPERATURE WITH VARIOUS DEGREES OF AIR DISCHARGE

Gauge Pressure, p s i	Saturated Steam Complete Air Discharge °C	Two-Thirds Air Discharge °C	One Half Air Discharge °C	One Third Air Discharge °C	No Air Discharge °C
5	109	100	94	90	72
10	115	109	105	100	90
15	121	115	112	100	100
20	126	121	118	115	100
25	130	126	124	121	115
30	135	130	128	126	121

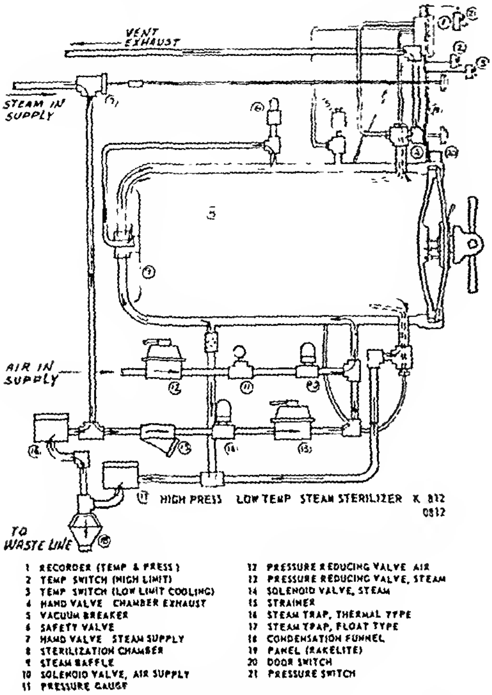

Fig. 2.—A comprehensive description of the dual-control autoclave.

adjusts the flow of steam and compressed air into the chamber upon demand, utilizing steam at 132° and air at 32°. By this device prolonged surges of steam and air, which would influence temperature and pressure stability, are thereby prevented. The dual-control autoclave is capable of operation within a range of 0–30 p. s. i. and 50–127°.

The autoclave is equipped with a timer which controls the duration of the sterilizing cycle once the desired temperature of the container contents has been reached. At the point when the chamber temperature has reached the autoclaving temperature, sufficient air under pressure is automatically admitted to obtain the predetermined total pressure. At the end of the sterilization cycle, the timer permits the autoclave to gradually exhaust steam at a rate which can be preset and calls for ambient air which enters the chamber through a sterile filter, thereby preventing a drop in chamber pressure during the cooling cycle. However, the cooling cycle can be terminated at will by manually opening the exhaust valve The chamber temperature and pressure as well as the temperature of the contents in the containers are constantly recorded during the sterilization cycle.

Materials

The jelly used in this study was N, N-dimethyl-N′-benzyl-N′ (alpha-pyridyl) ethylenediamine hydrochloride (Pyribenzamine anesthetic jelly 2%) containing 0.5% chlorobutanol as preservative and formulas of this jelly with the following modifications: (*a*) minus chlorobutanol, (*b*) minus Pyribenzamine HCl, (*c*) minus both chlorobutanol and Pyribenzamine HCl.

The ophthalmic solutions used were 2-(4-*tert*-butyl-2,6-dimethyl) benzyl-2-imidazoline hydrochloride (Otrivin 0.05%) containing 1:100,000 phenylmercuric acetate as preservative, as well as the same solution minus phenylmercuric acetate and, as a control, 0.9% sodium chloride solution.

B. cereus spores.

Collapsible aluminum tubes.

Amber, screw-capped, 15-ml. bottles with heat resistant screw caps made of black phenolic mineral composition.

Procedure

Sterilization in Dual-Control Autoclave.—A number of filled tubes of jelly and bottles of ophthalmic solutions were subjected to several temperatures for periods of time as indicated in Tables III and IV. The pressure within the chamber as well as the temperatures of the chamber and container contents were recorded throughout the sterilization cycle. For comparative purposes, other filled containers were treated at varying temperatures in a conventional autoclave for thirty-minute periods.

Microbiological Testing.—The ophthalmic solutions and jellies to be sterilized in the dual-control autoclave were contaminated with a concentrated saline suspension of *B. cereus* spores prior to filling into the bottles and collapsible aluminum tubes, respectively. Thorough mixing was achieved by means of a Cenco electric stirrer, as evidenced by replicate platings. After subjecting the tubes and bottles to the conditions of autoclaving as described in Tables III and IV, plate counts for survivors and sterility tests in fluid thioglycollate medium were performed.

TABLE III.—STERILIZING CONDITIONS FOR PHARMACEUTICAL JELLIES IN COLLAPSIBLE TUBES

Autoclave	Temperature, °C.	Gauge Pressure, p. s. i.	Duration, min
Dual control	95	10	30
	95	15	30 and 45
	105	10	30
	105	15	30 and 45
	105	20	30
	105	25	30
	105	30	30
	115	10	30
	115	15	30 and 45
	115	20	30
	115	25	30
	115	30	30
Conventional	100	..	30
	105	3	30
	115	10	30

TABLE IV.—STERILIZING CONDITIONS FOR OPHTHALMIC SOLUTIONS IN SCREW-CAPPED BOTTLES

Autoclave	Temperature, °C	Gauge Pressure, p. s. i.	Duration, min.
Dual control	95	10	30
	95	15	30
	105	15	30
	105	20	30
	105	25	30
	115	15	30
	115	25	30
	115	30	30
Conventional	100	..	30
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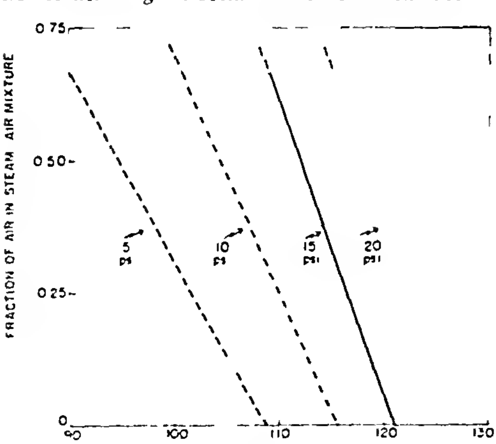

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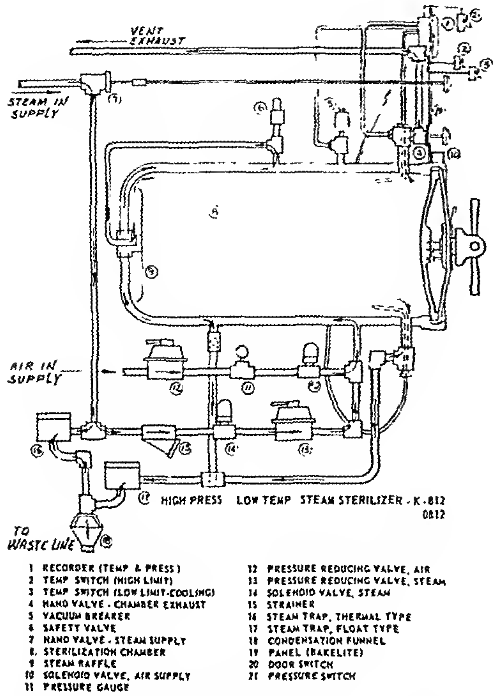

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	115	10	30
	115	15	30 and 45
	115	20	30
	115	25	30
	115	30	30
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	95	15	30
	105	15	30
	105	20	30
	105	25	30
	115	15	30
	115	25	30
	115	30	30
Conventional	100	..	30
	105	3	30
	115	10	30

Pyribenzamine Anesthetic Jelly 2%.—The contents of each tube were placed into a sterile beaker and mixed thoroughly. Duplicate 1.0-Gm. portions of jelly were diluted in trypticase soy broth, plated on trypticase soy agar, and survivors determined. In addition, duplicate 1.0-Gm. portions of material were placed into 200-ml. amounts of fluid thioglycollate medium and incubated at 37° for fourteen days. The absence of growth indicated sterility.

Otrivin Ophthalmic Solution 0.05%.—Duplicate 1.0-ml. portions of solution were diluted in broth and then plated in trypticase soy agar containing 0.03% thioglycollic acid (thioglycollic acid was used to neutralize possible carry-over of phenylmercuric acetate) and survivors determined. Additional 1.0-ml. portions of solution were transferred to 200-ml. amounts of fluid thioglycollate medium and the bottles incubated for fourteen days at 37°. The absence of growth at the end of this period of time indicated sterility.

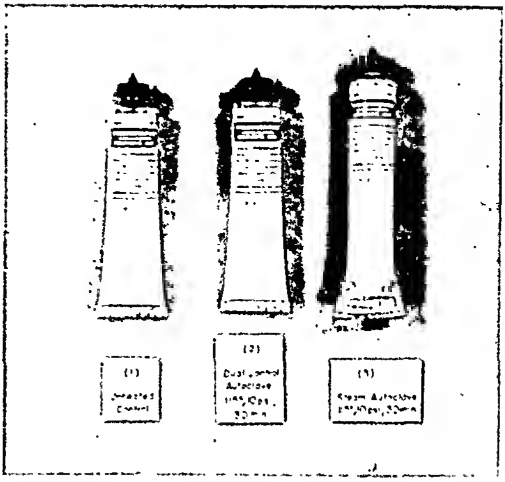

Fig. 3.—A photo of the appearance of the tubes containing Pyribenzamine anesthetic jelly before and after sterilization in the dual-control autoclave and conventional steam autoclave.

RESULTS AND DISCUSSION

Antibursting and Antileaking Effect of Air Pressure During Steam Autoclaving.—Since the water content in the jelly is approximately 85% and the ophthalmic preparation is an aqueous solution, the pressure in the tubes and bottles at the varying autoclaving temperatures can be essentially attributed to the vapor pressure of water at these respective temperatures. The effect of the components in the formula upon the pressure within the containers is insignificant since they are present in negligible quantities as compared to water. Since the vapor pressure of a liquid is a constant quantity at any temperature and is independent of the amount of liquid present (3), it is possible to obtain the vapor pressure of water from the critical tables.

Through the utilization of the dual-control autoclave, it is possible to prevent swelling or the bursting of collapsible tubes of pharmaceutical jellies as well as the leakage of solution from screw-capped bottles by the adjustment of the pressure within the chamber to approximate that of the contents within the containers.

The physical condition of the filled tubes of jelly was found essentially unaltered when treated in the dual-control autoclave under the conditions outlined in Table III. This absence of deleterious effect is also shown in Fig. 3. Careful inspection of these jelly-filled tubes treated below 20 p. s. i. at 105° and below 25 p. s. i. at 115° showed barely perceptible expansion. However, at pressures of 20 p. s. i. and above at 105° and at a pressure of 25 p. s. i. and above at 115° no evidence of any deleterious effects could be noted.

The glass containers as well as the screw caps used for the ophthalmic solution bore no visible evidence of change upon sterilization in the dual-control autoclave at any of the autoclaving conditions noted in Table IV. As already indicated, a special heat resistant cap of black phenolic mineral composition was used in this study.

Figure 3 also illustrates the undesirable effects that occur within the conventional autoclave in which the chamber pressure is strictly a function of steam alone. From a physical standpoint, the effect on the tubes has been disastrous.

Microbiological Studies. The products used in these studies were Pyribenzamine anesthetic jelly preserved with 0.5% chlorobutanol and Otrivin ophthalmic solution preserved with 1:100,000 phenylmercuric acetate. Previous experiences had indicated that the presence of these preservatives renders it possible to sterilize these products at temperatures substantially below 121°. Since the dual-control autoclave permits the treatment of filled collapsible tubes and screw-capped bottles to a variety of temperatures without deleterious effects to the containers, experiments were performed in which these products were heated in this autoclave at varying temperatures and pressures.

In one experiment a quantity of jelly was contaminated with approximately 180,000 *B. cereus* spores per Gm. of material. The contaminated jelly was filled into one-ounce collapsible tubes and crimped. At the Wilmot Castle Laboratories, the tubes were treated in the dual-control autoclave under varying conditions of temperature and pressure and then returned to our laboratories for sterility tests and survivor counts. The results of this experiment are shown in Table V and indicate that the product is effectively sterilized at a temperature as low as 95°.

TABLE V.—INFLUENCE OF TEMPERATURE, PRESSURE (AIR PLUS STEAM), AND DURATION ON THE STERILIZATION OF PYRIBENZAMINE ANESTHETIC JELLY

Run No.	No. of Tubes Tested	Temperature, °C.	Pressure, p. s. i.	Time, min.	Results
1	3	95	10	30	Sterile
2	3	95	15	30	Sterile
3	3	95	15	45	Sterile
4	3	105	10	30	Sterile
5	3	105	15	30	Sterile
6	5	105	20	30	Sterile
7	5	105	25	30	Sterile
8	5	105	30	30	Sterile
9	3	115	10	30	Sterile
10	3	115	15	30	Sterile
11	5	115	20	30	Sterile
12	5	115	25	30	Sterile
13	5	115	30	30	Sterile

Unheated control 180,000 organisms/Gm. Inoculum 300,000 organisms/Gm.

It was also felt desirable to subject to these same conditions tubes of jelly devoid of either chlorobutanol, or Pyribenzamine HCl, or both, contained in collapsible aluminum tubes. According to experiments conducted in our laboratories earlier, Pyribenzamine HCl is capable of exerting a mild but measurable preservative action and it was, therefore, desirous to establish the role this substance plays in the sterilization, at marginal temperatures, of *B. cereus* contaminated jelly. In addition, it was also desirable to determine the effect of the presence of chlorobutanol on the effectiveness of submarginal temperatures in destroying microorganisms in Pyribenzamine anesthetic jelly. Accordingly, four different batches of jelly were prepared. They consisted of (*a*) the complete formula, (*b*) the product minus the chlorobutanol, (*c*) the product minus Pyribenzamine, and (*d*) the product minus both chlorobutanol and Pyribenzamine. The results of this experiment to determine the influence of Pyribenzamine and chlorobutanol on the sterilizing capabilities of the autoclave at 95° and 105° are shown in Table VI.

From these results it is evident that, whereas the complete formula can be sterilized at a temperature of 95°, the removal of either chlorobutanol or Pyribenzamine HCl renders it impossible to sterilize the jelly at this temperature. It is only at 105° that these formulations can be effectively sterilized. Thus chlorobutanol and Pyribenzamine play important roles in the sterilization of this preparation at temperatures below 105°.

Similar experiments were performed with preserved (phenylmercuric acetate 1:100,000) and unpreserved Otrivin ophthalmic solutions. In addition, and for reference purposes, a solution of 0.9% sodium chloride was also tested.

According to data presented in Table VII, screw-capped bottles of Otrivin ophthalmic solutions, unpreserved or preserved with 1:100,000 phenylmercuric acetate, as well as 0.9% sodium chloride solution were sterilized within thirty minutes at temperatures of 95° and above and at pressures of 10 p. s. i. and greater.

Temperature Relationship Between Tube and Bottle Contents and Autoclave Chamber.—During the various autoclaving experiments, the temperature differential throughout the sterilization and subsequent cooling cycles of the chamber and the material being treated was recorded. This was accomplished through the use of thermocouples attached to a strip chart recorder and recordings made of the internal container temperature, external container temperature, and the chamber temperature during each experimental run.

It is evident from Figs. 4 and 5 that the rise and fall of the temperatures within the tubes and bottles lag behind the temperature outside of the containers and of the chamber. Comparing Fig. 4 for the tubes of jelly with that of Fig. 5 for the bottles of ophthalmic solution, it is evident that the temperature lags are greater for the bottle contents than for the tube contents. This can be essentially ascribed to the difference in the thermal conductance between aluminum and glass. The thermal conductance for aluminum has been reported as 0.49 cal. $cm.^{-1}$ $sec.^{-1}$ $°C.^{-1}$ while that for glass as approximately 0.0018 cal. $cm.^{-1}$ $sec.^{-1}$ $°C.^{-1}$ (4). The above figures are representative for sterilization at 115°.

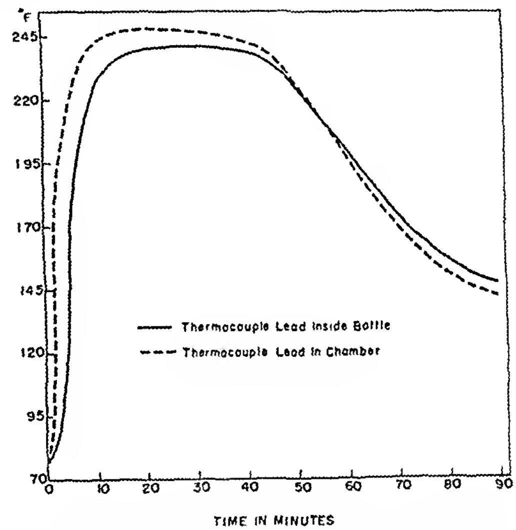

Fig. 4.—A plot of the temperature in the chamber and of the tube contents during a sterilization run in the dual-control autoclave at 115° and 25 p. s. i.

TABLE VI.—INFLUENCE OF DIFFERENT TEMPERATURES, PRESSURES (STEAM PLUS AIR), AND DURATION OF TREATMENT ON THE STERILIZATION OF CONTAMINATED JELLIES OF DIFFERENT COMPOSITIONS

Formulation	No. of Tubes Tested	Temperature, °C.	Pressure, p. s. i.	Time, min.	Survivors, org./Gm.	No of Tubes Sterile
Complete	3	95	15	30	0	3
	3	95	15	45	0	3
	3	105	15	30	0	3
	2	(Unheated controls)			120,000	0
Minus chlorobutanol only	4	95	15	30	<10	2
	4	95	15	45	<10	2
	4	105	15	30	0	4
	3	(Unheated controls)			350,000	0
Minus Pyribenzamine only	4	95	15	30	<10	0
	4	95	15	45	<10	3
	4	105	15	30	0	4
	3	(Unheated controls)			304,000	0
Minus chlorobutanol and Pyribenzamine	3	95	15	30	<10	0
	4	95	15	45	<10	1
	4	105	15	30	0	4
	3	(Unheated controls)			250,000	0

Inoculum—350,000 spores/Gm.

TABLE VII.—INFLUENCE OF TEMPERATURE, PRESSURE (STEAM PLUS AIR), AND DURATION ON THE STERILIZATION OF CONTAMINATED OPHTHALMIC SOLUTIONS

Formulation	No. of Samples Tested	Temperature, °C	Pressure, p. s. i.	Time, min.	Survivors, org./Gm.	No. of Samples Sterile
Otrivin ophthalmic solution 0.05% with phenylmercuric acetate 1:100,000	4	95	10	30	0	All sterile
	4	95	15	30	0	All sterile
	4	105	15	30	0	All sterile
	4	105	20	30	0	All sterile
	4	105	25	30	0	All sterile
	4	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	4	115	30	30	0	All sterile
	3	(Unheated controls)			117,000	. . .
Otrivin ophthalmic solution 0.05% without preservative	4	95	10	30	0	All sterile
	4	95	15	30	0	All sterile
	4	105	15	30	0	All sterile
	4	105	20	30	0	All sterile
	4	105	25	30	0	All sterile
	4	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	1	115	30	30	0	All sterile
	3	(Unheated controls)			81,000	
Sodium chloride solution 0.9%	4	95	10	30	0	All sterile
	4	95	15	30	0	All sterile
	3	105	15	30	0	All sterile
	4	105	20	30	0	All sterile
	1	105	25	30	0	All sterile
	1	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	5	115	30	30	0	All sterile
	3	(Unheated controls)			88,000	

Inoculum 125,000 organisms/Gm.

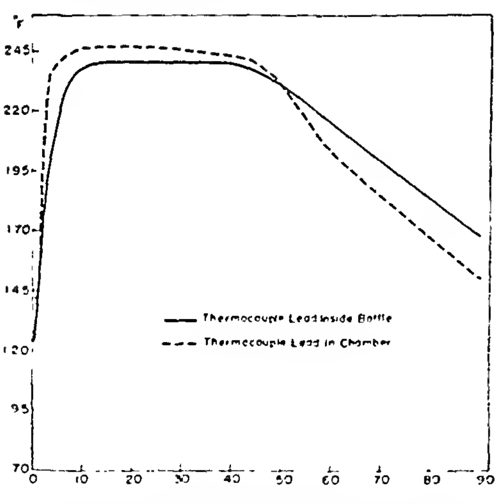

Fig. 5.—A plot of the temperature in the chamber and of the bottle contents during a sterilization run in the dual-control autoclave at 115° and 25 p. s. i.

However, similar curves are also obtained at temperatures of 95 and 105°. The lag during the cooling portion of the curve has been observed to become greater as the temperature of autoclaving is increased.

Because there appears to be a lag in the temperature within the containers during the sterilization cycle with respect to chamber temperature, a lag which undoubtedly would increase as the volume of the containers is increased, it is essential that the sterilization cycle should start when the proper temperature within the container is attained and not according to the chamber temperature.

SUMMARY AND CONCLUSIONS

It has been shown that through the use of air during the steam sterilization and subsequent cooling cycle, the bursting of collapsible aluminum tubes filled with a pharmaceutical jelly and the leakage of solution from screw-capped bottles of ophthalmic solution have been prevented. These effects have been achieved by modifying a conventional steam autoclave into a dual control autoclave in which the pressure and temperature in the sterilizer can be controlled independently of each other.

Pharmaceutical jellies have been sterilized in the dual control autoclave under various conditions of temperature and pressure without deleterious physical effects upon the filled collapsible tubes.

Ophthalmic solutions in screw-capped bottles have been sterilized in the dual-control autoclave under various conditions of temperature and pressure without deleterious effects upon the caps and bottles and with no leakage of solution.

Data have been presented dealing with the temperature differential of the chamber and container contents during the sterilization and subsequent cooling cycles. The thermal conductivity of the container composition plays an important role in this temperature differential.

The influence of the preservative chlorobutanol and the active constituent Pyribenzamine in Pyribenzamine anesthetic jelly, on the sterilizing capabilities of the submarginal temperatures have been investigated.

The effectiveness of submarginal temperatures on the sterilization of Otrivin ophthalmic solutions has been determined in the presence and absence of the preservative phenylmercuric acetate.

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(1) Lewith, S., *Arch. exptl. Pathol. Pharmakol.*, 26, 341 (1890).
(2) Reddish, G. F., "Antiseptics, Disinfectants, Fungicides, and Sterilization," 2nd ed., Lea and Febiger, Philadelphia, Pa., 1957, p. 272.
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A Note on the Synthesis of Some Alkyl and Alkylamine Esters of N,N′-Diphenylphosphorodiamidic Acid*

By LINDLEY A. CATES and TONY E. JONES

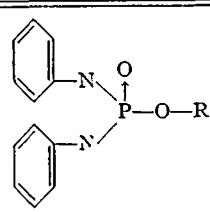
ESTERS OF CARBOXYLIC ACIDS in which basic groups are introduced in both acid and alcohol residues (i.e., dibutoline) and those in which the basic group is located only in the acid portion of the molecule are known to have antispasmodic activity. Since alkyl and alkylamine esters of a disubstituted phosphorous acid, N,N′-diphenylphosphorodiamidic acid, appear to possess some structural relationships to these carboxylic acid esters, a series of eight such compounds was prepared for study as possible antagonists of acetylcholine and as inhibitors of cholinesterase. These compounds, listed in Table I, were synthesized by the treatment of monochlorophosphoryl dianilide (7) with various alcohols, according to the method of Michaelis (1).

Previously synthesized esters of N,N′-diphenylphosphorodiamidic acid are the ethyl (1), phenyl (2), *p*-chlorophenyl (3), *o*-carboxyphenyl (4), 2-chloroethyl (5), and a series of six complex esters prepared by the method of Zetzsche and Buttiker (6). No alkylamine esters have been reported until the present work.

EXPERIMENTAL

Three grams of sodium metal was allowed to react with 150 cc. of the appropriate anhydrous alcohol. To the sodium alcoholate was added 8.0 Gm. of monochlorophosphoryl dianilide with an accompanying increase in temperature. The mixture was stirred for twenty minutes and the sodium chloride removed by filtration. The clear filtrate was added to 500 cc. of water with the formation of a dense, white precipitate. The precipitate was collected on filter paper and washed with water until the washings were neutral. Recrystallization from hot, dilute ethanol yielded the pure product. In the case of the *sec*-butyl derivative, the filtrate was concentrated to a syrupy mass and placed in a refrigerator overnight. The crystals that formed were collected, washed with water, and recrystallized from hot dilute ethanol. Repeated recrystallization of compound No. 8 failed to produce a completely pure product.

TABLE I.—ALKYL AND ALKYLAMINE ESTERS OF N,N′-DIPHENYLPHOSPHORODIAMIDIC ACID



No.	R	Yield, %	M. p., °C.[a]	Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	Methyl	40	108–109	59.53	59.31	5.76	5.66
2	*n*-Propyl	61	101–103	62.05	61.18	6.59	6.62
3	iso-Propyl	60	149–150	62.05	61.18	6.59	6.95
4	*sec*-Butyl	31	111–113	63.14	63.28	6.95	7.23
5	β-Diethylaminoethyl	55	122–123	62.23	61.78	7.25	7.25
6	β-Dimethylaminoethyl	35	161–162	60.17	59.64	6.94	6.97
7	β-Dimethylamino-α-methylethyl	19	145–147	61.24	61.03	7.25	7.24
8	β-Amino-α-methylethyl	13	192–194	59.00	60.29	6.60	5.86

[a] All melting points are uncorrected.

REFERENCES

(1)
(2)
(3)
(4) 31, 2177(1898).
(5) G., *Chim. & ind. Paris*, 64, 287(1950).
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	4	105	20	30	0	All sterile
	4	105	25	30	0	All sterile
	4	115	15	30	0	All sterile
	4	115	25	30	0	All sterile
	4	115	30	30	0	All sterile
	3	(Unheated controls)			117,000	....
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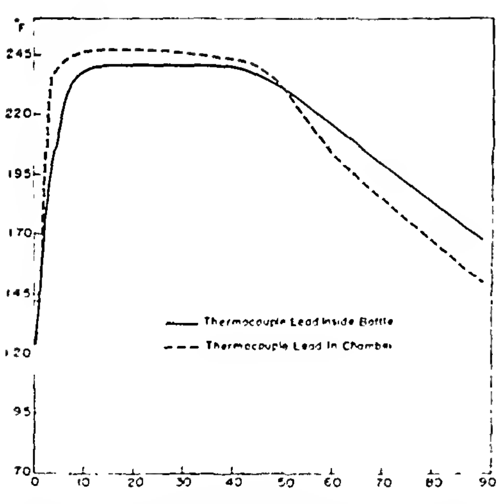
Inoculum 125,000 organisms/Gm.



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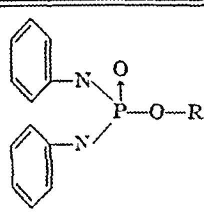

No.	R	Yield, %	M. p., °C.[a]	Analysis Carbon Calcd.	Analysis Carbon Found	Analysis Hydrogen Calcd.	Analysis Hydrogen Found
1	Methyl	40	108–109	59 53	59.31	5.76	5.66
2	*n*-Propyl	61	101–103	62.05	61.18	6 59	6 62
3	iso-Propyl	60	149–150	62.05	61.18	6.59	6 95
4	*sec*-Butyl	31	111–113	63.14	63.28	6.95	7 23
5	β-Diethylaminoethyl	55	122–123	62.23	61.78	7.25	7.25
6	β-Dimethylaminoethyl	35	161–162	60.17	59.64	6.94	6.97
7	β-Dimethylamino-α-methylethyl	19	145–147	61.24	61.03	7.25	7 24
8	β-Amino-α-methylethyl	13	192–194	59 00	60.29	6.60	5 86

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A Note on the Use of Citric Acid and Tartaric Acid Buffers in the Extraction of Solanaceous Alkaloids by Centrifugation*

By DANIEL P. N. TSAO

WITT, *et al.* (1), and Semchetti and Youngken (2) recommended McIlvaine's citric acid buffer for the extraction of alkaloids from small quantities of belladonna and hyoscyamus leaves but found it less satisfactory for the complete extraction of the total alkaloid of stramonium leaf. This note describes a modified method in which a centrifugation procedure is recommended as a substitute for filtration during the extraction of solanaceous alkaloids from the plants.

EXPERIMENTAL

The experiments are divided into two parts: (*A*) comparison of the efficiency of the alkaloidal extraction when citric and tartaric acid buffers are employed according to the general method of Witt, *et al.* (1, 2), and (*B*) comparison of the above results with those obtained when a centrifugation procedure is adopted in the place of the general method described previously.

Part A.—Assays of total alkaloids of belladonna and stramonium leaf material were carried out according to the procedure of Witt, *et al.* (1, 2). Citric and tartaric acid buffers were used as menstruum. The tartaric acid buffer consisted of 75 parts of 0.1 *M* tartaric acid and 25 parts of 0.2 *M* dibasic sodium phosphate.

Part B.—The centrifugation method was carried out in the following manner: a 0.5 Gm. sample of a fine powdered leaf material was placed in a 50-ml. Erlenmeyer flask. Twenty-five ml. of the specified menstruum (citric or tartaric acid buffer) was added, the flask was stoppered and the plant material was then shaken in this solution for fourteen to sixteen hours by a mechanical shaker. Thereafter, the mixture was centrifuged and filtered into a dry glass container. Three milliliters of the filtrate was made alkaline with ammonium hydroxide T.S. and then shaken vigorously in a separatory funnel with 25 ml. of chloroform for three minutes. The remainder of the procedure followed that described by Witt, *et al.* (1, 2).

RESULTS AND DISCUSSION

Results of analyses of belladonna and stramonium leaf material are shown in Tables I and II. It was observed that the average of total alkaloids in belladonna leaves extracted by citric and tartaric acid buffers which were subsequently separated from the plant material by filtration were 0.415 ± 0.039 and 0.447 ± 0.039%, respectively. The averages of total alkaloids in the same leaf material extracted by the same two buffers but separated by centrifugation were 0.429 ± 0.015 and 0.467 ± 0.022%, respectively. In stramonium, the total alkaloids extracted by citric and tartaric acid using filtration process were 0.183 ± 0.012 and 0.201 ± 0.025%, respectively, and by the centrifugal procedure were 0.206 ± 0.022 and 0.225 ± 0.018%, respectively.

TABLE I.—ALKALOID ASSAY OF BELLADONNA LEAVES BY CITRIC ACID AND TARTARIC ACID BUFFERS, CENTRIFUGATION *vs.* FILTRATION

	—Citric Acid—		—Tartaric Acid—	
Sample	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %
1	0.375	0.390	0.400	0.417
2	0.385	0.392	0.405	0.451
3	0.390	0.421	0.420	0.458
4	0.400	0.441	0.475	0.479
5	0.457	0.500	0.480	0.492
6	0.480		0.500	0.500
Average	0.415	0.429	0.477	0.467
S. D.	±0.039	±0.015	±0.039	±0.022

[a] Calculated as atropine.

TABLE II.—ALKALOID ASSAY OF STRAMONIUM BY CITRIC ACID AND TARTARIC ACID BUFFERS, CENTRIFUGATION *vs.* FILTRATION

	—Citric Acid—		—Tartaric Acid—	
Sample	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %	Filtration Alkaloids,[a] %	Centrifugation Alkaloids,[a] %
1	0.167	0.180	0.170	0.192
2	0.175	0.187	0.185	0.203
3	0.178	0.192	0.190	0.213
4	0.185	0.192	0.195	0.215
5	0.190	0.196	0.215	0.222
6	0.201	0.202	0.250	0.226
7		0.219	. .	0.229
8		0.220		0.210
9		0.221		0.255
10		0.218		0.266
Average	0.183	0.206	0.201	0.226
S. D.	±0.012	±0.022	±0.025	±0.018

[a] Calculated as atropine.

The tartaric acid buffer proved slightly more efficient for the extraction of solanaceous alkaloids from belladonna and stramonium leaf material than the citric acid buffer, but the increase was not significant.

The centrifugation method required about twenty minutes in comparison with three to five hours for the separation of the buffer from the plant mixture by the method of Witt, *et al.*, and Semchetti and Youngken (1, 2).

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The Colorimetric Determination of Lidocaine With *cis*-Aconitic Anhydride*†

By EDWARD G. FELDMANN and HENRY M. KOEHLER

The quantitative reaction between tertiary amines and *cis*-aconitic anhydride has been applied to the assay of microquantities of the local anesthetic lidocaine. The modified colorimetric method which is employed provides a particularly useful selective means for the assay of small samples containing this drug, which might arise from dosage form studies or biological investigations. The procedure detailed is quite simple in operation but yet shows relatively high precision and accuracy over the suggested range.

THE ANALYSIS of microquantities of the local anesthetic lidocaine (α-diethylamino-2,6-acetoxylidide) and its salts has long presented a problem to research workers Most methods which are presently available, such as acid-base titrations (1, 2), potentiometric titration (3), and quantitative nitration (4), require relatively large samples and are not suited to microanalysis Two procedures which have been employed as micromethods involve precipitation with reinecke salts (5, 6) or complex formation with methyl orange (7). Both of these methods however, suffer in that they are not too specific and practically any other amino compound present will give rise to a very significant degree of interference

Moreover, there are other limitations to each of these methods which further restricts their usefulness Reinecke salts often do not give quantitative precipitation, and in addition, relatively large quantities of the precipitate may be lost mechanically during the washing process, or significant amounts of excess reagent adhering to the precipitate may be carried over due to incomplete washing In the assay with methyl orange some of the sample may be lost during the step in which the ethylene dichloride sample solution is washed This is particularly a problem if the pH is not adequately controlled Furthermore, as Brodie and co-workers (8) have pointed out, "The sensitivity of the reaction in this solvent (ethylene dichloride) is limited by some solubility of free methyl orange in ethylene dichloride. . . ." This would, of course, give rise to some additional error.

In spite of the limitations listed, each of these procedures is useful in certain instances Even in these cases, however, particularly because of the questionable specificity of these methods, it would be highly advantageous to have another analytical procedure available—particularly one which might be more selective.

A simple, selective micromethod for the determination of lidocaine would be useful both in drug assay work, where it often is necessary to analyze less than a single dosage unit[1] (1 to 2 ml of a 1 to 2 per cent solution) of the anesthetic agent, or in the determination of the quantity of lidocaine present in small quantities of biological materials used in connection with pharmacological studies of the drug Simple ultraviolet spectroscopy, which has proved to be extremely useful in such cases when most other local anesthetic compounds are considered, is of limited value in the case of lidocaine because of its significantly lower absorption in this spectral range. The absorptivity of

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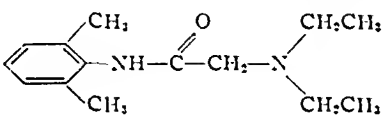
† Analysis of Local Anesthetics III For the preceding paper in this series see THIS JOURNAL, 48, 197(1959)

The authors wish to express their appreciation to Dr J Roy Doty for his helpful comments, and to Mrs Helen Jones for her assistance in preparing the manuscript

[1] Astra Pharmaceutical Products, Inc , markets solutions for injection containing lidocaine hydrochloride under the brand name of Xylocaine hydrochloride

an aqueous solution of lidocaine hydrochloride at its maximum wavelength, for example, is less than 5 per cent of that observed with procaine hydrochloride, butethamine hydrochloride, naepaine hydrochloride, or tetracaine hydrochloride at their respective wavelengths of maximum absorption. As a result, it is rather difficult to assay small quantities of lidocaine by ultraviolet spectroscopy; even in those cases where larger samples or greater concentrations of the drug are available, one must expect larger errors since impurities displaying absorption at the same wavelength give rise to a relatively greater degree of interference. This follows because it is not possible to reduce the effect of such impurities through dilution to the same extent as in the case of the other anesthetic compounds cited. For these same reasons, spectrophotofluorometry has also been of limited practical value (9).

Lidocaine is practically unique among the commonly used local anesthetic agents in that it does not contain either a primary or secondary amino group. Inspection of the structural formula for this compound, which is illustrated (I), shows that



I

chemically the only functional groups are an amide and a tertiary amine. Since amides historically do not lend themselves to microanalysis, a method of analysis based upon the tertiary amine was sought. Inspection of the literature shows that few methods, other than those which have been noted above as having been employed for lidocaine, are available for the determination of tertiary amines. Furthermore, these include no assay procedure of a truly micro nature.

Very recently, however, Sass and co-workers (10) reported a general method for the determination of tertiary aliphatic amines employing a reaction first described by Palumbo (11) as a qualitative procedure, and later extended by Cromwell (12) to the quantitative determination of trimethylamine in biological samples. This procedure appeared to hold promise of being potentially useful in the analysis of lidocaine. Subsequent experimental investigation in this laboratory showed that upon making several relatively minor modifications, a generally useful method for the determination of micro quantities of the anesthetic agent resulted. It should be noted, however, that in the assay of lidocaine contained in either dosage form samples or biological materials, the presence of other tertiary amines, which might be carried over in the toluene extraction, will give rise to interference when using this procedure.

EXPERIMENTAL

Chemicals.—Samples of lidocaine free base were generously supplied by Astra Pharmaceutical Products, Inc. *cis*-Aconitic anhydride was purchased from several chemical manufacturers. Melting points of the various samples and the colorimetric response of reagents prepared from these samples differed considerably, however. Uniformly good results with negligible blank readings were regularly obtained using samples purchased from the California Corporation for Biochemical Research. Reagent grade acetic anhydride (J. T. Baker) was routinely redistilled and only the fraction boiling at 138-140° was collected. Toluene, employed for the preparation of the reagent and also for dilution of standards and samples, was routinely purified because even reagent grade toluene, when used directly, gave rise to variable blank readings. The following purification method was found to yield toluene of uniformly high purity.

Five liters of reagent grade toluene (Merck) is shaken for several minutes in a large separatory funnel with about 300 ml. of concentrated sulfuric acid. The acid layer is withdrawn and the toluene is washed with two portions of water (200-300 ml.). The organic layer is transferred to a large reagent bottle, and a quantity (200-300 Gm.) of anhydrous sodium sulfate is introduced; the bottle is stoppered and shaken periodically over several hours. The toluene is then filtered and distilled; the fraction boiling at 109-111° is collected.

Reagent.—An accurately weighed 0.25-Gm. portion of *cis*-aconitic anhydride is transferred to a 100-ml. volumetric flask, and 40 ml. of redistilled acetic anhydride is introduced. The flask and its contents are swirled to effect complete solution; the flask may be warmed gently if necessary, and then cooled. The solution is diluted to volume with purified toluene and mixed. The solution is stored in a glass-stoppered bottle and allowed to age at room temperature for twenty-four hours prior to use. Under conditions of low humidity the solution appears to be stable for about two weeks.

Apparatus.—All glassware employed must be scrupulously clean and particularly free of contamination by any material which can act as a Lewis base. It should be noted that this includes certain types of detergents. A Beckman Model DK-2 recording spectrophotometer, with silica cells of 1-cm. light path, was employed to record the absorption spectra. The same instrument, with a model 92430 time drive attachment, was used in the color stability study. Absorbance values at peak wavelengths were checked, employing a Beckman DU spectrophotometer. All other absorption measurements, including those taken for the preparation of standard curves, were made using a Klett-Summerson colorimeter with a No. 54 filter.

Procedure.—*Preparation of Standard and Sample Solutions.*—An accurately weighed 120-mg. sample of pure lidocaine base is placed in a 100-ml. volumetric flask, the flask is filled to the mark with toluene, and mixed. A 5.0-ml. aliquot of this solution is transferred to a 50-ml. volumetric flask, diluted to volume with toluene, and mixed (standard solution A).

A 25-ml aliquot of solution A is similarly diluted to 50 ml. (standard solution B), and a 10-ml. aliquot of solution B is diluted to 50 ml (standard solution C) This dilution scheme provides standard solutions covering the upper limit (standard A; 120 μg per ml), the middle (standard B; 60 μg per ml), and the lower limit (standard C; 12 μg. per ml.) of the assay range combined with a conservative use of purified toluene and reliability in the accuracy of dilutions

An accurately measured aqueous or aqueous acid solution of the unknown lidocaine sample is made alkaline in a separatory funnel with ammonia T S and is promptly extracted with an accurately measured volume of toluene The bulk of the toluene layer is removed, centrifuged briefly, and an aliquot is withdrawn for dilution to the desired concentration so that readings will fall within the useable range.

Color Development—Samples of 2 0 ml each of the standard and unknown solutions are placed in 10-ml glass-stoppered volumetric flasks A 2 0-ml. sample of toluene is placed in another flask to serve as a reagent blank. To each flask is added 1 ml of the *cis*-aconitic anhydride reagent, the contents are mixed, and the flasks are loosely stoppered Each flask is heated in a boiling water bath for forty-five seconds and allowed to stand at room temperature for fifteen minutes The solutions are then diluted to volume with a solution containing 20% acetic anhydride and 80% toluene (v/v), mixed, and allowed to stand at room temperature for an additional fifteen minutes

Readings of color intensity are taken with the instrument set using toluene as the reference solvent The readings obtained with standard samples are graphed and the concentration of lidocaine in the unknown sample is determined from the resulting standard curve

RESULTS AND DISCUSSION

Extraction into Toluene—Analysis of aqueous solutions of lidocaine hydrochloride are dependent upon the completeness with which the lidocaine free base in such solutions is extracted with a single portion of toluene The completeness of extraction was checked by two methods In the first, a sample of standard lidocaine base was made up in aqueous hydrochloric acid and the color developed and measured after extraction, while another sample was dissolved directly in toluene and the color was developed and measured. The readings for equal concentrations were the same in each case, indicating that the entire sample of lidocaine carried on through The second method employed was a variation of the N F method (1) in which an aliquot of a single toluene extract was taken rather than pooling several chloroform extracts From the titration results it was again demonstrated that complete carry-over into toluene occurs with a single such extraction.

Reaction Involved.—Sass (10) has postulated that the reaction proceeds between *cis*-aconitic anhydride and a tertiary amine to form a complex ionic species The structure of the postulated product is such that it would be expected to present a number of resonance forms which serves to explain the highly colored nature of the complex The absorption curve in the visible region obtained with a typical sample of the colored lidocaine complex is shown in Fig 1. It can be seen from the figure that the absorption maximum occurs at about 540 mμ It should also be noted that the absorption of a reagent blank, similarly run, is very low at this wavelength Both of these absorption curves were taken against toluene as a reference solvent

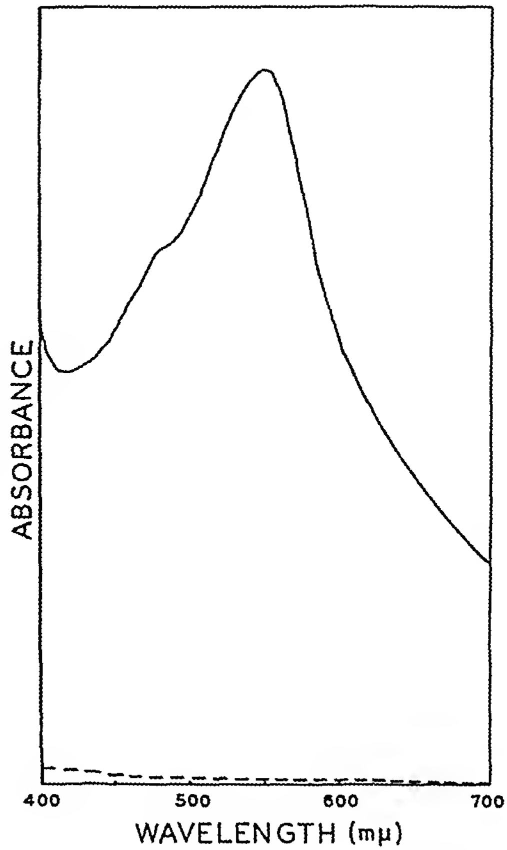


Fig 1.—Visible absorbance curves of color complex, —— lidocaine complex, - - - - reagent blank

Dilution with Acetic Anhydride-Toluene.—In some cases in which relatively large concentrations of anesthetic were employed, some precipitation of the colored lidocaine complex of *cis*-aconitic anhydride was noted if final sample dilution was made simply with toluene, as in the procedure suggested by Sass It was found, however, that use of a mixed solvent containing 20% acetic anhydride prevented precipitation even at anesthetic concentrations exceeding the useful range of determination Consequently, the use of this mixed solvent is recommended routinely in the procedure for purposes of final dilution after color development

Optimum Heating Period.—A series of lidocaine solutions containing the *cis*-aconitic anhydride reagent were heated for various time intervals to establish the optimum length of heating It was found that the most consistent, reproducible readings resulted when solutions were heated for more than thirty seconds and less than sixty seconds As a result, forty-five seconds is recommended

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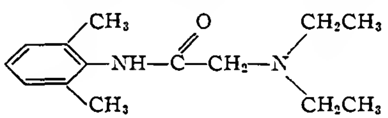

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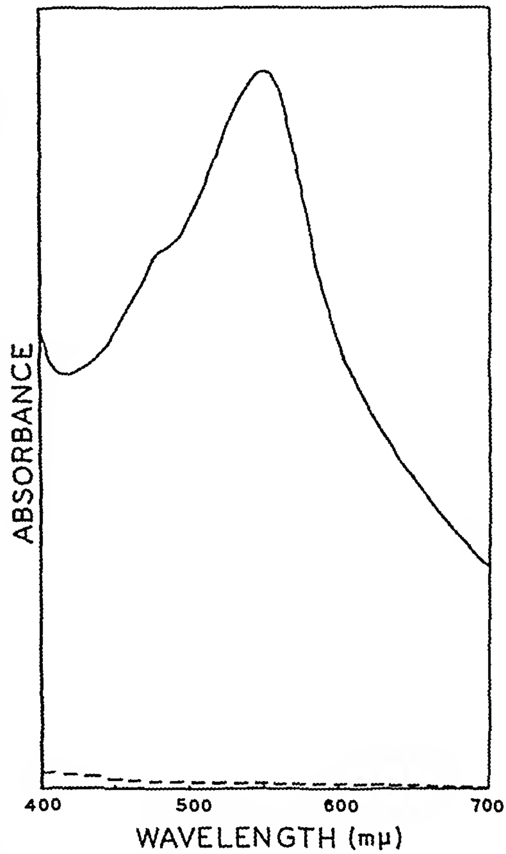

Fig. 1.—Visible absorbance curves of color complex; —— lidocaine complex; – – – – reagent blank.

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Optimum Heating Period.—A series of lidocaine solutions containing the *cis*-aconitic anhydride reagent were heated for various time intervals to establish the optimum length of heating. It was found that the most consistent, reproducible readings resulted when solutions were heated for more than thirty seconds and less than sixty seconds. As a result, forty-five seconds is recommended.

Stability of the Reagent.—As noted above, the reagent must be aged for twenty-four hours prior to use Reproducible readings were obtained with *cis*-aconitic anhydride solutions which were two weeks old, after two weeks readings tend to become gradually lower Since standard solutions are run simultaneously with unknown samples, this is not a critical consideration but it is not recommended that reagent solutions older than two weeks be employed routinely

Stability of Standard Lidocaine Solutions.—Gradually lower readings were obtained even with fresh reagent when the same toluene solutions of pure lidocaine base were used over a period of some time This would appear to be due to gradual degradation of the anesthetic in the solvent For this reason it is suggested that standard solutions of lidocaine base be prepared daily

Range of Anesthetic Concentration.—Colorimeter readings which were proportional to the anesthetic concentration were obtained with standard toluene solutions of lidocaine containing from 12 to 120 μg. per ml Since 2 ml of solution is employed in the procedure this would be equivalent to a total sample of 1.0×10^{-8} to 1.0×10^{-7} moles At concentrations higher or lower than this range the color response was found to be grossly nonlinear A graph of colorimeter readings plotted *versus* lidocaine concentrations is illustrated in Fig 2 It should be noted from the figure that over the suggested range there is an excellent straight-line relationship between color intensity and concentration

Stability of Color Produced.—After addition of the color developing reagent and subsequent heating, an initial fifteen-minute period of standing at room temperature is allowed to enable complete reaction to occur before the solutions are diluted to volume

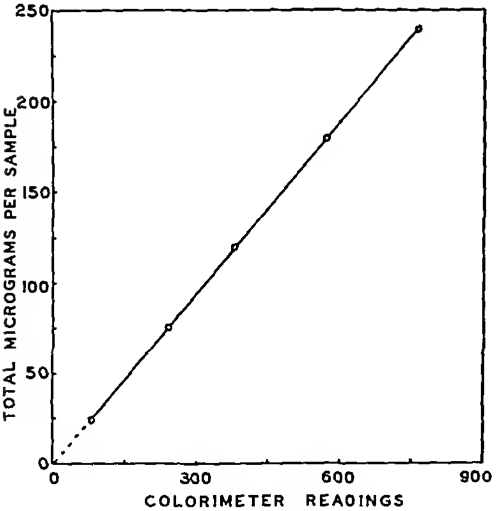

Fig 2—Typical standard curve prepared by plotting lidocaine base concentrations *versus* colorimeter readings obtained after color development

Experimentally, color intensities of several test samples were then taken over a two-hour interval using a recording spectrophotometer. It was noted that the color intensity drops rather sharply during the first few minutes, levels off for some five to fifteen minutes, and then continues to fall off very gradually for the balance of the time studied These results indicated that readings are most reproducible when taken fifteen minutes after the solutions have been diluted to volume.

Precision and Accuracy.—Some indication of the precision or reproducibility of the method can be seen by examination of Table I

TABLE I—COLORIMETER READINGS OF REPLICATE LIDOCAINE SAMPLES[a]

Sample	Reading	Sample	Reading
1	378	5	381
2	375	6	384
3	370	7	378
4	380	8	376

Mean—377 8 Standard deviation—4 2. Precision—1.1%

[a] Each sample contained a total of 120 μg lidocaine base

Eight replicates of a standard lidocaine solution were treated by the procedure described and the colorimeter readings obtained with the resulting solutions are given in the table

A stock solution of aqueous "synthetic commercial solution," prepared with accurately weighed portions of each ingredient, in the same concentrations as listed by the manufacturer's label, was made up as a standard solution which would simulate the commercial product Portions of this solution in varying volumes, some of which were as small as 1 ml, were then run through the toluene extraction, and after appropriate dilution were subjected to color development

The data from these experiments indicated that an accuracy of ± 2–3% can be anticipated from the method when the procedure is followed carefully and the cautions detailed herein, such as purity of solvents and glassware cleanliness, are rigidly followed.

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Determination of Epinephrine in the Presence of Degradation Products*

By TAKERU HIGUCHI, THEODORE D. SOKOLOSKI, and LOUIS C. SCHROETER

The chemistry of the presently official method of analysis for epinephrine has been closely examined. Results show that the official procedure may lead to formation of other acetylation products in addition to the expected triacetylepinephrine (TAE). Evidence is also offered showing that some oxidation products are acetylated and extracted. A modification is proposed based on chromatographic isolation of TAE in place of the time-consuming extraction step and close control of the acetylation step. Results of a comparative study of four possible assay methods for epinephrine are also presented.

Determination of epinephrine in the presence of its degradation products presents an unusually difficult analytical problem. Although the method of the U. S. P. XV (1) based on the work of Welsh (2) represents a major step[1] in solving the problem, it possesses certain unsatisfactory features. In the present work an attempt has been made to shorten and improve the official method. The basic method as proposed by Welsh consists of triacetylation of the drug, exhaustive extraction of the acetylated product with chloroform, and gravimetric determination of the extracted solids. The acetylation step was introduced to convert the unextractable, difficultly-soluble epinephrine base into the freely-soluble triacetyl form. Results of the present investigation show that in the official method the weighed product is usually not pure acetylated compound and this leads to variable errors. The present communication is concerned with modifications which appear to overcome the shortcomings of the official assay method.

EXPERIMENTAL

The following experimental conditions were generally observed in carrying out these studies:

Sample Preparation.—Epinephrine solutions containing chlorobutanol were transferred to a separatory funnel and extracted four times with equal volumes of reagent grade carbon tetrachloride. Five milliliters of the aqueous solution, representing about 5 mg. of epinephrine base, was transferred to a 50-ml. tall beaker and a stirring magnet added. Bisulfite or sulfite, if present, was oxidized by microiodine titration to the starch end point—slight excess of iodine was destroyed by the addition of 0.01 *N* sodium thiosulfate. Twenty-five milliliters of reagent grade isooctane was added to the beaker.

Acetylation.—The O^3, O^4, N-triacetyl derivative of epinephrine (TAE) was quantitatively prepared by introducing a total of 0.23 ml. acetic anhydride in divided portions and 435 mg. sodium bicarbonate to the epinephrine solution with rapid stirring. A typical schedule is shown below:

Acetic Anhydride, ml.	Sod. Bicarbonate, mg.	Stirring Time, sec.
0.13	435	60
0.05		60
0.05		60
Total 0.23	435	180

For studies made with varying time of reaction, approximately the same proportions of time was used for each step. About 100 mg. citric acid was added to buffer and stabilize the reaction mixture after completion of acetylation. The mixture was stirred while 5.6 Gm. of Celite 545[2] was added in portions to prepare a uniform slurry for chromatographic assay.

Chromatography.—Fourteen grams of Celite 545 wetted with 12.6 ml. of pH 4, 0.1 molar citrate buffer was slurried with isooctane and packed into a chromatographic column (2 × 45 cm.) in portions as described by Higuchi and Patel (3). Excess isooctane was drained from the column and the previously prepared slurried acetylated mixture was quantitatively transferred and packed into the prepared column. The reaction beaker was rinsed with a slurry of isooctane and 1 Gm. of Celite wetted with 0.9 ml. buffer; this was also packed into the column. The finished column was drained of isooctane and developed with chloroform. The first 10-ml. eluate was discarded, then exactly 50 ml. was collected for spectrophotometric or gravimetric determination. Actually several runs showed that essentially all of the TAE in the sample was contained between the 20th and 30th ml. of eluate, but additional volume was collected on either side of the peak to insure total recovery.

Spectrophotometry and Polarimetry.—The ultraviolet spectrum of the eluates was determined with a Cary recording or Beckman DU spectrophotometer. Solutions which exhibited low absorbances were concentrated from 50 ml., by evaporation, to 25 ml.

Determination of optical activity of the triacetyl derivative, when employed, was carried out by first evaporating combined eluates representing about 17 mg. of the derivative. The eluates were evaporated to dryness with gentle heat and dissolved in about

* Received December 29, 1958, from the School of Pharmacy, University of Wisconsin, Madison.
This project was in part conducted under a contract with Armed Forces Medical Procurement Agency, Brooklyn, N. Y., and was in part supported by a grant from Parke, Davis and Co., Detroit, Mich.

[1] The development of this general procedure by Welsh for epinephrine represents a very significant advance in the science of analytical chemistry. The theoretical rationale underlying the method probably has not been fully appreciated.

[2] Johns-Manville Corp.

0 5 ml of chloroform The chloroform solution was quantitatively transferred to a 1 dm micro polarimeter tube of known volume (1 65 ml) and the volume carefully adjusted with chloroform rinsings Measurements were made at 25° with a Zeiss-Winkel polarimeter, using sodium D line (589 mμ) light

Test for Stability of TAE Against Hydrolysis in the Internal Phase.—Stability of the triacetyl derivative to hydrolysis when present in the internal phase was tested by dissolving triacetylepinephrine in pH 3 90 and pH 5 58, 0 1 molar, citrate buffers and storing the solutions at 25° Aliquots of the solutions were slurried with Celite 545 and isooctane, as described above, and packed into prepared chromatographic columns Columns were developed with chloroform Extent of hydrolysis was determined by ultraviolet spectrophotometry of the eluate and gravimetric determination of its residue

Test for Recovery of TAE from Chromatographic Columns.—Recoveries from columns buffered at pH 4 with 0 1 molar citrate was tested with O^3, O^4, N triacetyl *l*-epinephrine, m p 94–95°, $[\alpha]_D^{25}$ −94 5 Solutions of triacetylepinephrine were slurried with Celite 545 and isooctane and packed into chromatographic columns The columns were developed with chloroform Triacetylepinephrine was determined by ultraviolet spectrophotometry of the eluate and gravimetric determination of the residue

Comparative Study.—Solutions containing epinephrine in the presence of degraded material were analyzed by ultraviolet spectrophotometry, a modified U S P XIV colorimetric procedure (4), the U S P XV acetylation procedure, and by this modified acetylation procedure Epinephrine solution was degraded by oxidation in a sealed system in which the oxygen atmosphere was maintained at 1 5 atmospheres for one hundred hours at 80° Analysis of the resulting black, insoluble, degraded product by the acetylation procedure indicated no epinephrine present The degraded product along with synthetic *l* epinephrine was used to prepare known test solutions

The solutions were buffered at pH 4 0 with 0 1 molar acetate, allowed to stand four hours, then filtered several times to remove all insoluble material Ultraviolet spectra of the solutions, diluted to equal 1 Gm epinephrine base in 11,200 ml, were recorded with a Cary recording spectrophotometer from 250 to 310 mμ The absorbance at the 280 mμ maximum was compared with a Beer's law plot

Solutions were also analyzed by a modified U S P XIV (4) ferro citrate colorimetric procedure Five milliliters of the solution was diluted to 25 ml with 1 500 sodium bisulfite solution, a 5 ml aliquot was transferred to a 25 ml volumetric flask containing 10 ml of 1 500 sodium bisulfite solution To the solution was added 0 5 ml of ferro citrate and 5 ml of buffer solution prepared as described in the U S P XIV The volume was adjusted to 25 0 ml and absorbance determined at 530 mμ in a Bausch and Lomb model 20 colorimeter after thirty minutes color development Absorbances were compared with a Beer's law plot and the results tabulated

The U S P XV assay and the modified acetylation procedure described in the prior section were employed in comparative analyses of the solutions The solutions were diluted for the official assay with equal volumes of pH 4, 0 1 molar acetate buffer Thirty milliliters of diluted solution was acetylated and the weight of chloroform soluble acetylated product determined Ultraviolet spectra of those amorphous residues which gave gravimetric recoveries in excess of 100% were determined in 50 ml chloroform solution Optical rotation of 5 ml chloroform solutions of the residues was determined with a Zeiss Winkel polarimeter at 25° with sodium D line light, using a 1 dm micro tube (Table IV) Analytical values obtained with the U S P and the modified procedure refer to *l* epinephrine determined at O^3, O^4, N triacetyl *l*-epinephrine

RESULTS AND DISCUSSION

Experimental evidence obtained from these studies strongly indicates that the method of assay for epinephrine as described in the U S P XV is based partially on uncertain assumptions Close analysis of the reaction system does not point to straightforward quantitative formation of triacetyl epinephrine from the catecholamine under the official procedure Prolonged reaction of the drug with acetic anhydride apparently leads to production of other reaction products in addition to the major product, TAE This tendency not only interferes with the gravimetric determination of the acetylated material but also with analytical interpretations based on the optical activity of the isolated reaction products

Formation of another product at the expense of TAE is suggested by results of chromatographic analysis of the acetylation product When the acetylated reaction product, subjected to varying reaction times, was analyzed for its triacetyl content on a column, the results shown in Fig 1 were obtained The TAE shown on the figure (marked spectrophotometric) were determined spectrophotometrically following chromatographic isolation The data suggest that quantitative conversion of epinephrine to the triacetyl (TAE) derivative appears to occur within the first two minutes Longer reaction time seems to favor further reaction to give a significant amount of uncharacterized acetylation product, hereafter referred to as peracetyl compound Decrease in actual yield of TAE with continued reaction was not attributable to hydrolysis of the formed derivative since concurrent gravimetric determinations on the same eluates showed approximately 100% apparent recovery of chloroform soluble product calculated as TAE, as also shown in Fig 1

It is apparent from Fig 1, that the official gravimetric method yields fairly acceptable results primarily because the other product seems to have similar solubility characteristics and is extracted along with TAE It may be concluded, nevertheless, that a prolonged reaction period leading to formation of an end product other than TAE will tend to give high values in the official procedure

There existed a possibility that the apparent low recovery of TAE may be partly attributable to possible hydrolysis of the product during its chromatographic isolation Experimental tests indicate that this is not the case, as shown in Table I The triacetyl derivative was found to be stable towards

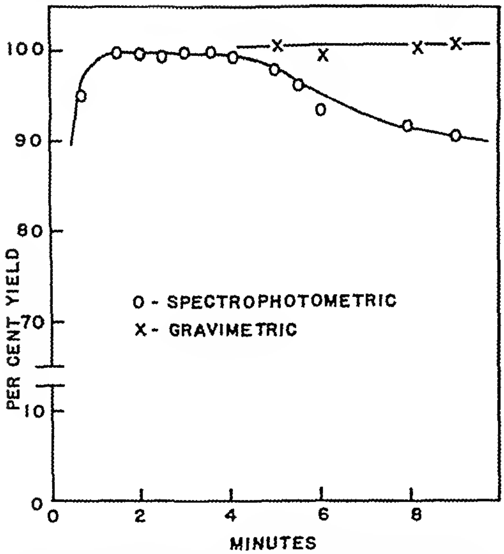

Fig. 1.—Apparent yield of triacetylepinephrine as function of reaction time. The circles represent results obtained by spectrophotometric determination on chromatographically isolated TAE. The crosses are results of direct gravimetric determination on total chloroform extracts.

hydrolysis for at least twenty-four hours when dissolved in pH 3.9–5.6 citrate buffers. Results of a direct test of recovery of triacetylepinephrine from chromatographic columns buffered at pH 4 with 0.1 molar citrate buffer are shown in Table II. The citrate buffered internal phase was found to give good separation of the acetylation mixture, triacetylepinephrine being quantitatively eluted and degraded products being retained.

Direct extraction of products of acetylation may lead to recovery of not only triacetylepinephrine but also certain acetylated forms of oxidized epinephrine as well as the reaction products which seem to result from exhaustive acetylation. The difficulty in reproducing the same degree of acetylation and extraction is evident in Fig. 2. The two upper spectral

TABLE I.—STABILITY OF O^3, O^4, N-TRIACETYL-*levo*-EPINEPHRINE IN 0.1 MOLAR CITRATE BUFFER SOLUTIONS AT 25°

pH	Residence Time, Hr.	Added, mg.	Found,[a] mg.	Recovery, %
3 90	0.25	76.1	76 3	100.3
3 90	24 0	76.1	78.3	102.8
5 58	0.25	68 7	69 1	100.5
5 58	24.0	68.7	69.7	101 5

[a] Determined gravimetrically following chromatographic isolation

TABLE II.—RECOVERY OF O^3, O^4, N-TRIACETYL-*levo*-EPINEPHRINE FROM CHROMATOGRAPHIC COLUMNS[a]

Column No	Added, mg	Found,[b] mg	Recovery, %
1	17 3	17.6	101 7
2	17 3	17 2	99 4
3	12 6	12 4	98 4
4	14 9	14.9	100 0

[a] Internal phase buffered at pH 4 with 0.1 molar citrate.
[b] Determined gravimetrically.

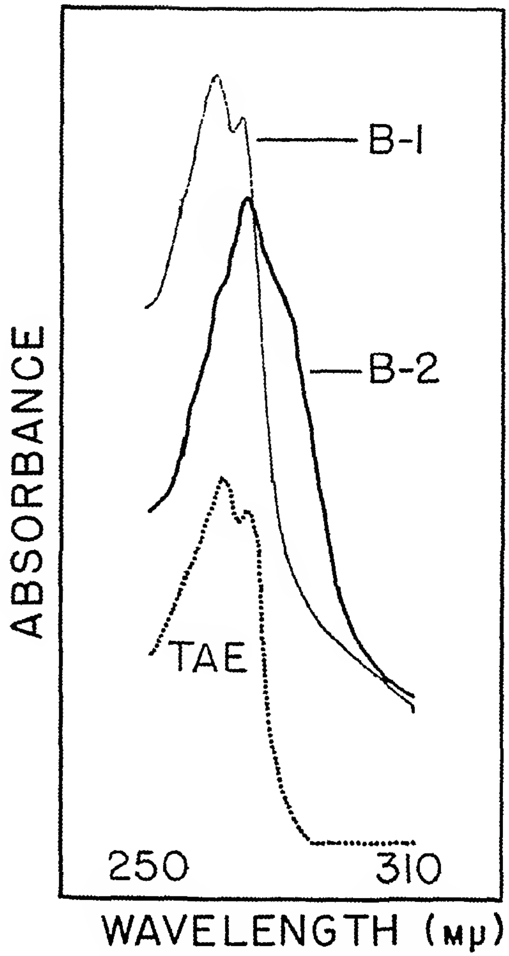

Fig. 2.—UV Spectra (B-1, B-2) of residues obtained in duplicate analyses of solution B (0.10% epinephrine, 0.10% degraded material) by U. S. P XV procedure. Lower curve (TAE) is the ultraviolet spectrum of O^3, O^4, N-triacetyl-l-epinephrine isolated by chromatography of residues

curves were obtained on extracts from duplicate analyses following the official procedure on a sample containing 0.1% epinephrine and 0.1% degraded epinephrine. The lower curve was found following chromatographic isolation of TAE from these runs.

The probable presence of acetylation product(s?) other than TAE, which tends to give high results gravimetrically, is not as serious as the interference by the degraded products. The actual loss of a significant portion of triacetyl compound by further reaction with acetic anhydride is over-compensated by the fact that the higher molecular weight peracetyl product is quite readily extracted by the official procedure. The formation of the peracetylation product, however, casts some doubt as to the validity of the polarimetric determination made directly on the extraction residue.

To test the relative merits of several analytical procedures available for determination of epinephrine in aqueous solution, analytical determinations were made by four independent methods on four

samples containing degraded epinephrine with apparent zero epinephrine content along with fairly pure drug. The methods studied included (*a*) direct ultraviolet absorption determination on the original solution, (*b*) modified U. S P XIV colorimetric method based on the use of ferro-citrate solution, (*c*) U. S. P XV gravimetric method, and (*d*) the modified U. S. P. XV method based on acetylation time of three minutes with mechanical stirring and chromatographic isolation of triacetylepinephine from the reaction mixture The results are shown in Table III.

Limitation of direct ultraviolet spectrophotometry to analysis of such degraded solutions is evident in Fig 3. The upper spectral curves were obtained by direct ultraviolet spectrophotometry of degraded epinephrine solutions. The lower spectral curves represent corresponding triacetylepinephrine derivatives of the solutions prepared by the modified U. S. P. XV assay employing chromatographic isolation of the derivative. Values reported from the colorimetric procedure in Table III were the best obtainable after pretreatment of solutions with activated charcoal and filtration to remove color of degraded epinephrine. Results reported for both the ultraviolet and colorimetric method should properly apply only to the catechol chromophore or presence of phenolic hydroxyls. The methods are no more discriminating than this and tell nothing about the rest of the molecule or proportions of isomers. The official U. S P. method gave high results in every instance as expected. The modified procedure, on the other hand, appears to give valid answers in every case.

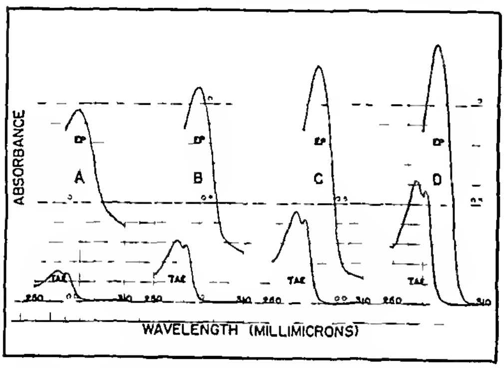

Fig. 3 —Ultraviolet spectra of several epinephrine solutions containing varying amounts of degraded products The curves marked EP were obtained by measurements directly on fixed dilutions of these solutions. The lower curves marked TAE refer to measurements on TAE fraction isolated by chromatography. The compositions of the solutions were

Sample No.	Epinephrine, %	Degraded Epinephrine, %
A	0 050	0 150
B	0 100	0 100
C	0 150	0 050
D	0 200	0 000

Polarimetric determinations on the acetylated extract following the official procedure gave, as expected, erroneous but interesting results. This is evident in Table IV. Because the recovered weight of the acetylation product was always much higher than theoretical for all samples containing degraded epinephrine the official method of calculation is, of course, meaningless. It is interesting to note, however, if all of the optical activity is attributed to *l*-triacetylepinephrine and that no other optically active compound is present, a rather nice agreement with theory is obtained. Since in the official procedure TAE is totally contained in the extract, such a result may be expected if the net optical effect of peracetylation on the system was small. This is true because in these particular samples no racemization existed For regular samples, however, such an assumption cannot safely be made and the modified procedure presented in this report must precede any polarimetric determination.

RECOMMENDATION

It is recommended that the modified procedure described in this report be adopted as the official

TABLE III.—COMPARISON OF ASSAY METHODS IN ANALYSIS OF DEGRADED EPINEPHRINE SOLUTIONS

	Composition, mg /100 ml		Epinephrine Found, mg /100 ml			
Solutions	Degraded Material	Synthetic *l*-Epinephrine[a]	U V	Colorimetric	U S P XV,[b] Gravimetric	Modified Procedure[c]
A	150	50	146	62	81.4	49 3
B	100	100	166	106	129 0	100.5
C	50	150	180	156	162 5	148 5
D	.	200	199	202	199 0	197.5

[a] Commercial high purity epinephrine with estimated purity above 98%
[b] Values based on gravimetric determination of residue ep = wt of residue × 0 5923
[c] Values reported as *levo*-epinephrine

TABLE IV.—GRAVIMETRIC AND POLARIMETRIC VALUES OBTAINED IN U. S. P. XV ASSAY

	Wt. of Acetylated Product, Gm					*l*-Epinephrine, mg. in 30 ml. Dilution		
Soln.[a]	Calcd.	Found	Recovery, % Gravimetric	α^b	$(\alpha)^{25°}_D$	Present	Found[c]	Found[d]
A	0 0127	0.0206	162	−0 23	−56 0	7 5	9 8	7 3
B	0 0253	0.0327	129	−0 46	−70 5	15 0	17 1	14 5
C	0 0380	0.0412	108	−0 72	−87 8	22 5	23 8	22 7
D	0 0507	0 0504	99 5	−0 95	−94 3	30 0	30 1	29 8

[a] 15 ml of solution diluted to 30 ml. with pH 4, 0 1 molar acetate buffer for analysis (See Table III for composition)
[b] Determined in 1-dm. micro tube.
[c] Calculated with expression· Wt. ep /30 ml = $[0\ 5 + 0\ 5(\alpha)^{25}_D/-93] \times [(\text{Wt Residue})(0\ 5923)]$.
[d] Calculated with expression: Wt. ep./30 ml. = $30 \times (\alpha^b - 0.95)$.

method for determination of epinephrine. The procedure appears to give valid results in all instances where it has been applied. It is not based on compensatory errors arising in the system. The method is theoretically sound in that results are directly related to quantitative conversion and isolation of the drug component into a definitely established derivative. Substitution of a chromatographic step in lieu of the exhaustive extraction in the official procedure, moreover, leads to an appreciable saving in operational time.

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The Solubility and Complexing Properties of Oxytetracycline and Tetracycline III*

Interactions in Aqueous Solution With Model Compounds, Biochemicals, Metals, Chelates, and Hexametaphosphate

By TAKERU HIGUCHI and SANFORD BOLTON†

The present study extends the investigation of complexing tendencies of oxytetracycline to (*a*) a series of isomer, multifunctional aromatic compounds, (*b*) certain biochemicals, (*c*) calcium and magnesium ions, both free and as chelates, and (*d*) hexametaphosphate. The relative importance of grouping and their location on the binding tendency has been demonstrated. Measurements carried out on purines, DNA, B vitamins, etc., show that such biochemicals are, in general, relatively strong complexing agents. Data on alkali earths suggest that complexed drugs may still undergo chelation. Hexametaphosphate appears to undergo very slight, if any, complexing reaction with either tetracycline or oxytetracycline at pH values between 5 and 8.

THE ABILITY of the tetracycline antibiotics to form complexes with organic substances as well as metal ions has been established in previous studies (1–4). In the present communication the binding properties of tetracycline have been subjected to further examination in an effort to obtain a broader picture of the conditions necessary for complex formation. More specifically, model complexing agents were chosen to shed additional light on factors which may produce the highest degree of binding. The interaction of oxytetracycline with some compounds of biological interest was also investigated on the basis that these substances contain appropriate polar centers and that these systems might possibly represent a mechanism of action of oxytetracycline *in vivo*. In addition, the binding behavior of metal chelates for organic molecules and interactions of oxytetracycline and tetracycline with sodium hexametaphosphate were examined.

These studies were not particularly undertaken to find new complex species of tetracyclines but rather to provide further insight into the general phenomenon of molecular binding. The antibiotic was chosen for the purpose of this investigation because (*a*) it possesses a number of functional groups conducive to complex formation as indicated by earlier studies and (*b*) because of its great medicinal importance. The binding and associative properties of the drug are, also, of fundamental interest in that its microbiological and pharmacodynamic actions may be strongly affected by such behaviors.

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STRUCTURAL CONSIDERATIONS

Because of the intricacy of these reactions and the complexity of the oxytetracycline molecule, it seems advisable, initially, to contemplate some factors which may affect these interactions. The extent of complex formation may be considered to be dependent, in part, on a series of competitive reactions. Accordingly, the intramolecular and intermolecular forces present in oxytetracycline would present a definite barrier to the formation of complexes. The low water solubility as well as the comparatively low melting point of oxytetracycline are probably manifestations of intramolecular hydrogen bonding. The hydroxy and carbonyl groups attached to carbons 10 and 11 and 12 and 1 are ideally situated for this type of bonding (Fig. 1). The fact that oxytetracycline forms a stable hydrate suggests a strong water-oxytetracycline bond which would interfere with an interaction of another species at the bonded sites.

Similarly, the complexing agent itself may be in-

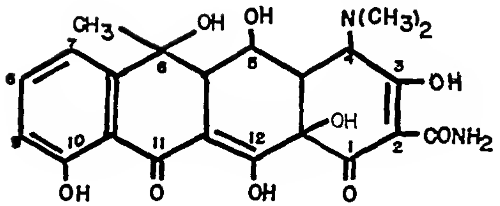

Fig 1—Oxytetracycline

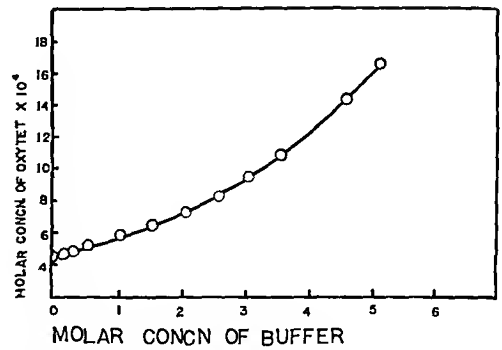

Fig 2—Phase diagram showing the effect of acetate buffer on the apparent solubility of oxytetracycline at 25°, pH 5

volved in a series of competitive reactions An attraction for itself or water would hinder its tendency to complex with oxytetracycline The fact that the complexing tendencies of the tetracyclines are greater in certain nonaqueous solvents (2), where at least competitive solvent interactions are lessened, seems to substantiate the above viewpoints These competing reactions cannot be separated and measured in such a complex system, but the physical properties of the substances involved can present an insight into the relative magnitudes of these attractive forces

EXPERIMENTAL

Reagents.—Oxytetracycline dihydrate and tetracycline trihydrate were prepared by methods suggested by Gans and Higuchi (1) The purity was checked by solubility analysis All other compounds were generally redistilled, recrystallized, or were of the highest purity commercially available Sodium hexametaphosphate was used directly as supplied by Charles Pfizer and Co Laboratories

Procedure.—The experimental procedure was essentially the same as that used by Gans and Higuchi (1) An excess of antibiotic was equilibrated with 10 cc of a solution of known concentration of complexing agent by shaking for eighteen hours at 25° The solutions were maintained at pH 5 by use of a suitable buffer, and 0 1% sodium bisulfite was added as an antioxidant In some cases, where the complexing agent was especially labile to oxidation, i e, aminophenols, dihydroxy benzenes, and ascorbic acid, 1% sodium bisulfite was added and equilibration was carried out in an atmosphere of nitrogen Clear aliquots were then appropriately diluted with pH 5 buffer for spectrophotometric analysis

It should be noted in this respect that the condition of constant pH imposes certain restrictions on the experiment At pH 5, oxytetracycline exists as a zwitterion and, therefore, the interaction form of oxytetracycline is necessarily limited to this species Similarly, the acid-base ratio of any acidic or basic complexing agent will be fixed by the pH In the absence of other factors, a homologous series of acids, for example, should show different degrees of activity depending on their ionization constants if either the acid or base species is active Also, the buffer concentration has a definite effect on the solubility of oxytetracycline as shown in Fig 2 This solubilization phenomenon was kept to a minimum by using a conveniently low buffer concentration

Analytical Methods.—The amounts of oxytetracycline and tetracycline in solution were measured by convenient dilution in pH 5 buffer and subsequent spectrophotometric analysis utilizing the peaks at 353 mμ and 355 mμ, respectively In certain cases, interference in this region of the spectrum by the complexing agent was unavoidable Appropriate blank corrections were made at various wavelengths and checked by measuring the absorption of the solutions at these wavelengths It was found that Beer's law relationship for oxytetracycline held at wavelengths from 350–380 mμ In the analysis of oxytetracycline riboflavin interaction, the minimum in the U V spectrum of riboflavin at 303 mμ was used to check the results obtained at 353 mμ

Salicylaldehyde was found to interfere with the analysis in a manner which could not be blanked out The solution after equilibration, in this instance, was made acid with hydrochloric acid and then extracted with three equal portions of ether Most of the salicylaldehyde was removed in this manner The small amount of oxytetracycline extracted was calculated and a correction made in the final analysis This procedure was found to be accurate with solutions of known concentration of oxytetracycline and salicylaldehyde

RESULTS AND OBSERVATIONS

The general type interaction exhibited by lower concentrations of various compounds with oxytetracycline in aqueous solutions is shown in Fig 3 Soluble complexes appeared to have formed in all cases studied In the presence of excess crystalline drug, the apparent amount of the antibiotic in solution appears to increase often linearly with the concentration of the complexing agent For such a system it is convenient to assume formation of a one to one complex and to calculate an equilibrium constant

$$K_1 = \frac{(TX)}{(T)(X)} \qquad \text{(Eq 1)}$$

where (X) is the concentration of free complexing agent, (T) is the concentration of free oxytetracycline, and (TX) is the concentration of complex The details of this calculation may be found in a paper by Higuchi and Zuck (5)

Figure 4 illustrates the effect of higher concentrations of many complexing agents It is obvious that the order with respect to complexing agent is increasing at higher levels Equilibrium constants

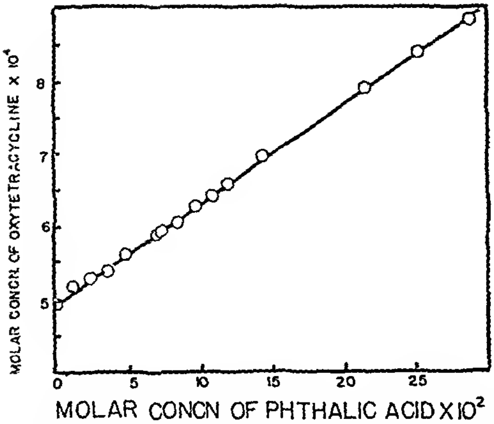

Fig 3.—Phase diagram showing the effect of phthalic acid on the apparent solubility of oxytetracycline at 25°, pH 5.

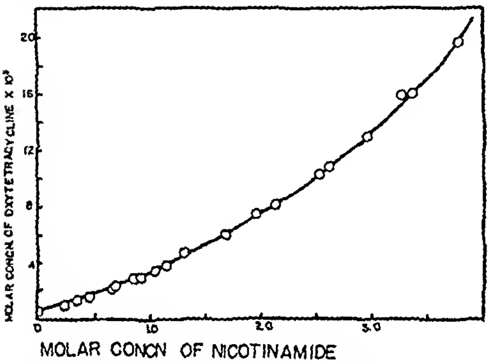

Fig. 4.—Phase diagram showing the effect of nicotinamide on the apparent solubility of oxytetracycline at 25°, pH 5.

were calculated for higher order interactions in the following manner:

$$K_2 = \frac{(TX_2)}{(X)(TX)} \qquad \text{(Eq. 2)}$$

$$K_3 = \frac{(TX_3)}{(X)(TX_2)} \qquad \text{(Eq. 3)}$$

Let

$$K_0 = \frac{(\text{Total Complex})}{(T)(X)}$$

$$= \frac{(TX) + (TX_2) + (TX_3)}{(T)(X)}$$

$$= \frac{(TX)}{(T)(X)} + \frac{(TX_2)}{(T)(X)} + \frac{(TX_3)}{(T)(X)}$$

$$= K_1 + K_1K_2(X) + K_1K_2K_3(X)^2$$

Rearranging,

$$\frac{K_0 - K_1}{K_1(X)} = K_2 + K_2K_3(X) \qquad \text{(Eq. 4)}$$

A plot of $K_0 - K_1/K_1(X)$ *vs.* (X) results in a straight line with slope K_2K_3 and intercept K_2. Typical results are shown in Fig. 5. In the cases of

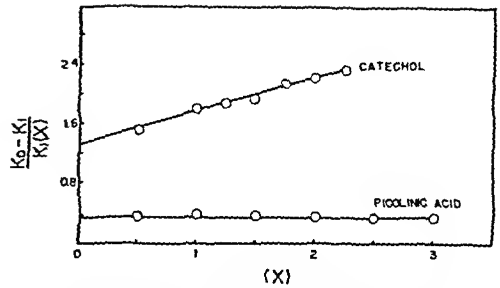

Fig. 5.—Plot illustrating the calculation of second and third order equilibrium constants.

m-hydroxybenzoic acid, salicylic acid, and pyrogallol, the solubility curves were described by K_1 and K_3 only, where K_3 equals $(TX_3)/(TX)(X)^2$. A modification of the above procedure was used to calculate K_3.

Although it cannot be shown that these equilibria are actually occurring in solution, these constants can accurately describe the solubility curves. To calculate the total concentration of oxytetracycline in solution at a particular concentration of complexing agent, the following method suffices:

$$\text{Total oxytetracycline in solution} = (T) + (TX) + (TX_2) + (TX_3) \qquad \text{(Eq. 5)}$$

Combining Eqs. 1, 2, and 3 with Eq. 4

$$\text{Total oxytetracycline in solution} = (T) + K_1(T)(X) + (T)K_1K_2(X)^2 + (T)K_1K_2K_3(X)^3 \qquad \text{(Eq. 6)}$$

Since (X) is essentially equal to the total amount of complexing agent added to the system and (T) is equal to approximately 5×10^{-4} *M*, Eq. 6 can be reduced to the following form:

$$\textit{Total oxytetracycline in solution} = 5 \times 10^{-4} \times [1 + K_1(X) + K_1K_2(X) + K_1K_2K_3(X)^3] \qquad \text{(Eq. 7)}^1$$

The slopes mentioned in Tables I, II, and III are merely the slopes of the straight lines obtained initially in the solubility measurements and are mentioned for convenience of comparison. The column headed "Complexing Agent Added" indicates the maximum amount of complexing agent added to the systems in these studies.

Interactions of Oxytetracycline With Homologous Polar Aromatic Compounds.—The purpose of this study was to note the effect of acidity and the presence of various polar groups, as well as their positions, on the complexing tendency with oxytetracycline. Table I shows that of the monofunctional compounds tested the carboxy group seemed to be more effective than either the aromatic nitrogen in pyridine or the amino group in aniline. In the series of dihydroxybenzene derivatives, resorcinol showed the greatest activity. The aminophenols and pyridine carboxylic acids showed a similar pattern: the *meta* isomer was stronger than the *para*, which in turn had a higher slope than the *ortho* isomer. Salicylic acid complexed most strongly in the series

[1] In the cases of pyrogallol, salicylic acid, and *m*-hydroxybenzoic acid where only K_1 and K_3 were necessary to describe the interaction, Eq 7 reduces to:
Total Oxytet. $= 5 \times 10^{-4} [1 + K_1(X) + K_1K_3(X)^3]$
where $K_3 = (TX_3)/(X)^2(TX)$

TABLE I.—INTERACTION BETWEEN OXYTETRACYCLINE AND VARIOUS AROMATIC COMPOUNDS AT pH 5 AND 25°

Compound	Slope × 10^2	Liters/Mole K_1	K_2	K_3	Molar Concn. Complexing Agent Added
Phenol	0.12	2.4	...	...	0.11
Aniline	0.06	1.2	...	...	0.105
Benzoic acid	0.16	3 2	...	...	0.21
Pyridine	0.06	1.2	0.85	...	1.0
Resorcinol	0 225	4.5	1.00	0.45	4.05
Catechol	0 16	3.2	1.32	0.34	2.6
Hydroquinone	0.20	4.0	...	...	0.095
p-Aminophenol	0.10	2.0	...	...	0.12
o-Aminophenol	0.07	1.4	...	...	0.05
m-Aminophenol	0.13	2.6	. .	...	0.11
Salicylic acid	0 32	6 4	...	0.7	3.00[a]
p-Hydroxybenzoic acid	0 28	5.6	...	...	0.075
m-Hydroxybenzoic acid	0.28	5.6	...	2.3	0.41
2,4-Dihydroxybenzoic acid	0 56	11.2	0.45	...	0.48
2,5-Dihydroxybenzoic acid	0.50	10.0	...	...	0.48
2,5-Dihydroxybenzoic ethanolamide	0.54	10.8	...	...	0.38
3,4-Dihydroxybenzoic acid	0.41	8.2	0.70	...	0.52
3,5-Dihydroxybenzoic acid	0.44	8.8	. .	...	0.128
p-Aminobenzoic acid	0.18	3.6	..	...	0.084
o-Aminobenzoic acid	0.22	4.4	...	...	0.064
m-Aminobenzoic acid	0.17	3.4	...	...	0.03
Nicotinic acid	0.195	3.9	...	...	0.16
Nicotinamide	0.205	4.1	0.34	...	3.9
Isonicotinic acid	0.155	3.1	...	...	0.10
Picolinic acid	0.13	2.6	0.31	...	3.56
Benzaldehyde	0.14	2.8	...	...	0.030
m-Hydroxybenzaldehyde	0 26	5.2	...	...	0.078
Salicylaldehyde	0.14	2.8	...	...	0.037

[a] Calculated from data by Gans and Higuchi (1).

TABLE II.—INTERACTION OF VARIOUS SUBSTANCES WITH OXYTETRACYCLINE AT pH 5 AND 25°

Compound	Slope × 10^2	Liters/Mole K_1	K_2	K_3	Molar Concn. Complexing Agent Added
Ethyltheophylline	0.74	14.8	...	...	0.127
Gallic acid	0 46	9.2	...	...	0.115
2,3-Diketo-1,2,3,4-tetrahydroxy-quinoxaline	0.44	8.8	...	...	0.0017
Apresoline HCl	0.40	8.0	...	...	0.079
p-Aminosalicylic acid	0.39	7.8	...	...	0.12
Melamine	0.32	6.4	...	...	0.064
Quinoline-8-carbox. acid	0.27	5.4	...	...	0.024
PEG 4000	0.26	5.2	...	...	0.06
Pyrogallol	0.22	4.4	...	0.84	3.17
Maleic hydrazide	0.19	3.8	...	...	0.055
8-Hydroxyquinoline	0.18	3.6	...	...	0.017
Antipyrine	0.17	3.4	0.36	...	3.25
o-Phthalic acid	0.14	2.8	...	...	0.36
Monomethyloldimethylhydantoin	0.095	1.9	0.37	...	2.8
Pyridazine	0.09	1.8	...	...	0.26
Dimethylhydantoin	0.07	1.4	0.43	...	1.05
Oxazolidone	0.06	1.2	0.19	0.23	3.0
Barbital	0.06	1.2	...	...	0.042
PEG 1500W	0.06	1.2	...	...	0.18
3-Hydroxy-3-methyl-2-butanone	0.012	0.24	1.2	...	0.405
Malonic acid	0.01	0.2	...	...	0.96

of hydroxybenzoic acids and, similarly, the hydroxy derivatives of salicylic acid proved stronger than other dihydroxybenzoic acids tested. Again, in the aminobenzoic acid series, the *ortho* isomer showed greatest activity. It is only in the case of the hydroxybenzaldehydes that the *ortho* isomer seemed to be less active. In an attempt to correlate these results with complexing activity, relative acidity and the positions of relevant groups were considered.

A direct correlation between acidity (as measured by ionization constants) and activity is not apparent. This is most easily seen in the series of dihydroxybenzoic acids. At the pH of these experiments the dihydroxybenzenes are essentially all in the nondissociated form. It should also be noted in this respect that the slopes obtained for gentisic acid and nicotinic acid were slightly smaller than the slopes of their amide derivatives.

In each series of homologous compounds the influence on binding tendency of position isomerism is evident. In all cases, except the benzaldehyde series, the compounds with polar groups separated

TABLE III —INTERACTION OF VARIOUS BIOCHEMICALS WITH OXYTETRACYCLINE AT pH 5 AND 25°

Compound	Slope × 10²	Liters/Mole K_1	K_2	K_3	Molar Concn Complexing Agent Added
Riboflavin-5-phos Na	1.28	25 6			0 0014
Desoxyribonucleic acid	1 15	23 0			0 08
Adenylic acid	0 50	10 0			0 062
Thymine	0 31	6 2			0 037
Uracil	0 25	5 0			0 019
Pyridoxine HCl	0 23	4 6	0 26	0 6	1 03
Dimethyluracil	0 20	4 0	0 5		1 16
Alloxan	0 25	5 0			0 14
Thiamine HCl	0 225	4 5	0 6	0 8	1 02
Tryptophane	0 20	4 0			0 01
Cytosine	0 15	3 0			0 059
Creatinine	0 13	2 6	0 35		1 06
l-Histidine HCl	0 07	1 4	0 45		0 89
Ascorbic acid	0 06	1 2			0 057
Urea	0 02	0 4	0 52		7 3

by three carbon atoms seemed to be most active Thus, if a more stable complex can be formed when two polar groups are ideally oriented so as to fit receptor sites in the oxytetracycline molecule, an optimum positional effect would be expected. The apparent anomaly in the case of salicylaldehyde can be rationalized on the basis of the very strong intramolecular hydrogen bonding existing in this compound (6)

It was noted, also, that the complexing properties of these multifunctional compounds are approximately additive Accordingly, the slope of a hydroxybenzoic acid was close to the sum of the slopes of benzoic acid and phenol Figure 6 shows a plot of expected slope value, calculated strictly on an additive basis, *vs* the observed value It is seen that although the properties are not strictly additive, there is a linear relationship in the series of compounds where polar groups are separated by 3 and 4 carbons The low deviation from this additive effect in the aminophenols could be due to strong intermolecular associations in solution. The high deviations in the cases of the dihydroxybenzoic acids could be explained on the basis of a greater possibility of multiple point contacts, which would produce a more stable complex.

Interactions of Oxytetracycline With Other Simple Polar Molecules of Various Configurations.—Table II lists some additional compounds which were used in this study They were chosen not only to help elucidate the data on the acids but also to note the effect of different types of configurations on complexing activity.

8-Hydroxyquinoline, 8-carboxyquinoline, the quinoxaline derivative, and apresoline hydrochloride all showed greater activity than was expected Although the reason for this would be difficult to interpret, it should be noted that these substances all contain fused ring systems Andrews has mentioned that the stability of 1–1 complexes increases with the number of rings in the complexing agent (7).

Both *p*-aminosalicylic acid and gallic acid behaved approximately as expected The lower slope of gallic acid and pyrogallol seems to substantiate the importance of position of polar groups A similar observation was made by Sakai in an investigation of the effect of various benzene derivatives on the solubility of riboflavin (8) Phthalic acid exhibited relatively weak binding properties

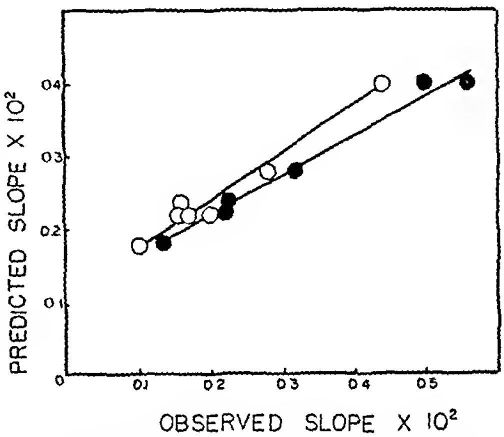

Fig 6 —Predicted slope value *vs* observed slope value where polar centers are separated by three carbons (●) and four carbons (O)

In contrast to aromatic compounds, purely aliphatic compounds show relatively low affinity for the antibiotic Both malonic acid, which contains the optimum "three carbon separation," and hydroxymethylbutanone had a very small effect on the solubility of oxytetracycline The behaviors of barbital, oxazolidone, and the hydantoins are also similarly weak This could be explained on the basis of *Pi* complexing or the necessity of a planar structure to obtain proper orientation

Interactions of Oxytetracycline With Compounds of Biological Interest.—Table III lists some metabolites whose configurations seemed to be appropriate for complex formation with oxytetracycline Oxytetracycline was found, for example, to have an effect on the excretion of B vitamins (9) and their activity (10) The investigation of these systems was also indicated on the basis that the biological action of oxytetracycline may conceivably involve association with essential metabolites *in vivo*.

Fused ring compounds such as desoxyribonucleic acid, adenylic acid, and riboflavin exhibited the strongest binding tendencies of the compounds in this series Tryptophane, which also falls into this class, showed similar high order of complexing ability Although other interesting biochemical compounds such as uric acid, folic acid, rutin, and quercitin were all considered, they were found to

so insoluble as to make any accurate observations impossible.

The results in the uracil series are interesting as they show the effect of substituted nonpolar groups on complexing activity. Uracil appears to be less active than thymine, and dimethyluracil less active, in turn, than uracil. The fact that these biochemicals do associate with oxytetracycline is not meant, of course, to infer that these interactions *per se* are important in the physiological activity of the antibiotic. Rather, the idea that these interactions do occur with such naturally occurring substances is of interest in that similar interactions may take part in the actions of such drugs.

Investigation of the Interaction of Metal Chelates of Oxytetracycline With Some Organic Molecules.—Figure 7 illustrates the effect of $CaCl_2$ and $MgCl_2$ on the solubility of oxytetracycline. Although the existence and properties of these chelates have been investigated elsewhere (11, 3), the possibility of metal chelates of oxytetracycline reacting in turn with an organic complexing agent seemed worthy of investigation.

The results of a typical experiment are shown in Fig. 8. The concentration of metal ion in the system was kept constant while the concentration of monomethyloldimethylhydantoin (MDH) was varied. The increase in solubility of oxytetracycline was noted beyond that calculated on an additive basis, i. e., independent interaction of metal and MDH added. A similar plot, keeping MDH constant and varying the concentration of metal ion, results, also, in a straight line. It is obvious from these plots that the increase in solubilization of oxytetracycline is proportional to the concentration of both metal and organic species. The relationship can be expressed by Eq. 8.

Increase in solubility beyond expected $=$ K (concentration of metal ion) X (concentration of comp. agent) (Eq. 8)

where K is an appropriate constant.

This relationship was tested at higher concentrations of metal and complexing agent, and the results as well as the "K" calculated from Eq. 8 are listed in Table IV. (In the oxazolidone-magnesium chloride system, a plot similar to Fig. 8 produced a curve which deviated from linearity at higher concentrations. An extrapolation of this curve was used to calculate the expected solubility of oxytetracycline at still higher concentrations.) This solubilization phenomenon is probably due to a new association species resulting from the interaction of the oxytetracycline chelate or the oxytetracycline-organic complexing agent association product with the free metal or organic compound.

These promising results suggested further investigation of similar systems involving a chelating agent, metal ion, and oxytetracycline. One of two results was expected: (*a*) the solubility of oxytetracycline would be reduced because of competition for the metal ion by the chelating agent, or (*b*) a new reaction would occur and the solubility of oxytetracycline would be significantly increased.

In the presence of ascorbic acid and magnesium chloride the solubility of oxytetracycline was increased only a small amount above that calculated on an additive basis. In view of the above discussion, this result was unexpected. However, a potentiometric titration of ascorbic acid in the presence of magnesium chloride compared to a similar titration in which magnesium chloride was replaced by sodium chloride of equal ionic strength demonstrated that no chelate formation occurs in this system, at least in the pH range 3–8. Thus, the small increase in solubility of oxytetracycline observed seems to be due only to the type of reaction exhibited in the three-component systems previously discussed.

It can be seen from Fig. 9 that the solubility of oxytetracycline is significantly reduced when EDTA is added to the system oxytetracycline-magnesium chloride. EDTA, alone, had a negligible effect on the solubility of oxytetracycline. Using the data of Schwarzenbach (12, 13), the amount of metal ion in solution in the presence of EDTA at pH 5 was calculated. The solubility of oxytetracycline observed did not correspond to the solubility expected from the above calculation but the presence of buffer as well as the competing oxytetracycline-metal interaction probably invalidates the use of Schwarzenbach's data here. It is to be noted that the decrease in solubility of oxytetracycline with rising pH

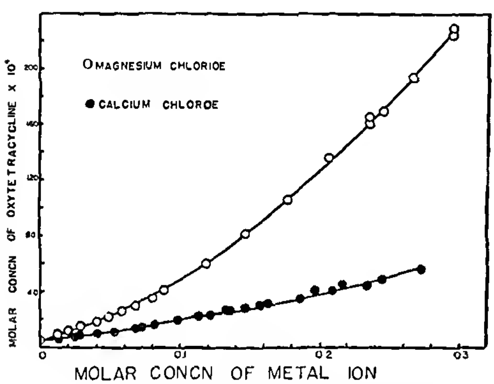

Fig. 7.—Phase diagram showing the effect of magnesium and calcium chlorides on the apparent solubility of oxytetracycline at 25°, pH 5.

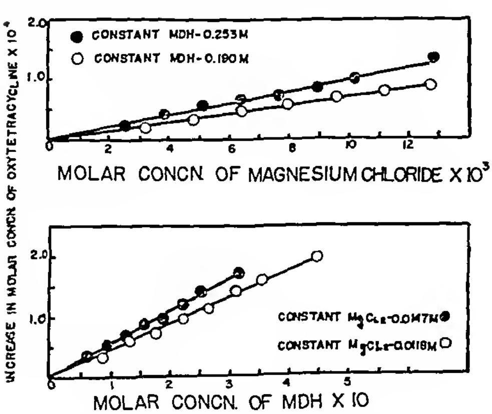

Fig. 8.—Plot showing the observed increase in the solubility of oxytetracycline beyond that calculated on an additive basis in the system magnesium chloride-monomethyloldimethylhydantoin-oxytetracycline at 25°, pH 5.

TABLE IV —INTERACTIONS OF CALCIUM AND MAGNESIUM IONS WITH ORGANIC COMPLEXING AGENT AND OXYTETRACYCLINE AT pH 5 AND 25°

Molar Concn Organic Compound		Molar Concn Metal Ion	Liters/Mole K	Calculated Increase in Molar Concn Oxytet × 10⁴	Observed Increase in Molar Concn Oxytet × 10⁴
Monomethyloldimethylhydantoin	0 675	Mg 0 038	0 038	21 3	21 2
	1 038	0 098		38 6	41 7
	1 210	0 118		54 3	57 2
	0 51	0 0494		9 6	7 5
	0 38	0 0365		5 2	6 7
Oxazolidone	0 35	0 0498	0 39[a]	7 8	8 3
	1 00	0 06		28 1	24 0
	1 05	0 076		40 5	39 7
	0 66	0 116		44 2	39 2
	1 44	0 106		80 7	84 3
Resorcinol	0 30	0 067	0 115	23 2	24 3
	0 448	0 158		81 5	80 0
	0 605	0 091		63 4	71 0
Ethyltheophylline	0 975	Ca 0 0997	0 22	21 4	22 0
	1 00	0 148		32 6	32 3

[a] At very low concentrations of Mg^{++} and oxazolidone

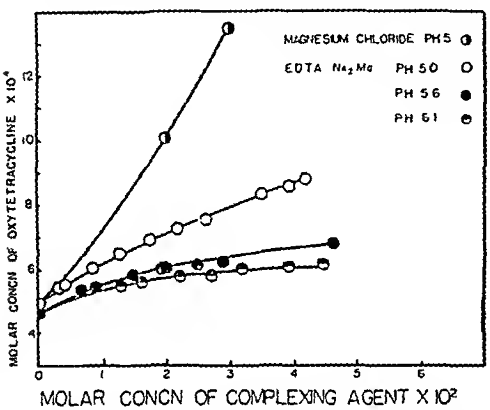

Fig. 9—Phase diagram showing the effect of Na_2Mg EDTA on the apparent solubility of oxytetracycline at 25° at several pH's. The effect of magnesium chloride at pH 5 is included for comparison

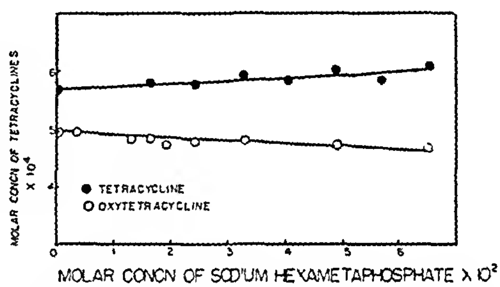

Fig. 10.—Phase diagram showing the effect of sodium hexametaphosphate on the solubility of tetracycline and oxytetracycline at 25°, pH 5

is to be expected if the decrease in solubility is merely due to the removal of metal ion by EDTA

Interaction Studies of Sodium Hexametaphosphate With Tetracycline and Oxytetracycline.—Recent commercial interest in tetracycline-hexametaphosphate combinations in various dosage forms prompted an investigation of a possible interaction in this system. A complex of sodium hexametaphosphate and tetracycline, isolated at pH 1 5 (14) and confirmation of the increased oral absorption of this combination (15, 16) have been reported in the recent literature

The results of solubility studies at pH 5 as depicted in Fig 10 strongly suggest that only minimal interaction occurs in these systems Similar results were obtained at pH 7 To check the possibility that these negative results were not due to certain anomalies in the experimental setup, such as interference by the buffers used, a variation was introduced The solutions of sodium hexametaphosphate and antibiotics were equilibrated in distilled water, the pH of each solution being checked The results of this study with tetracycline, including variations in pH with concentration of sodium hexametaphosphate, are shown in Fig. 11 Similar results were obtained with oxytetracycline The solubility and pH curves seem to parallel each other indicating that the change in solubility might be a function of pH only.

In conjunction with this study, the solubility of the tetracyclines was determined in the absence of buffer by extrapolating curves of antibiotic solubility *vs.* buffer concentration to zero buffer concentration at several pH's between seven and eight The results are shown in Fig 12. By subtracting the solubility as observed in Fig 12 from that observed in Fig 11 at that particular pH, the increase in solubility of tetracycline as a function of hexametaphosphate concentration was calculated The results, thus obtained, in both oxytetracycline and tetracycline systems are depicted in Fig 13 Although this plot seems to indicate that there is some interaction occurring, the errors inherent in the approximations introduced do not permit the use of these data as a means of analyzing the interaction.

The fact that comparatively little binding tendency was noted suggests that sodium hexametaphosphate must act in some other manner if the rate of absorption is actually increased in its presence. Thus, for example, increased oral absorption of this combination may be elicited through a membrane effect in addition to the sequestering behavior of sodium hexametaphosphate. The latter action probably accounts largely for the observed effect.

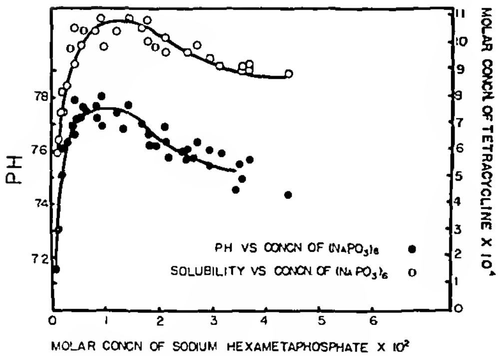

Fig 11 —Diagram showing the effect of sodium hexametaphosphate on the solubility of oxytetracycline and the pH of the resulting solutions in the absence of buffer at 25°

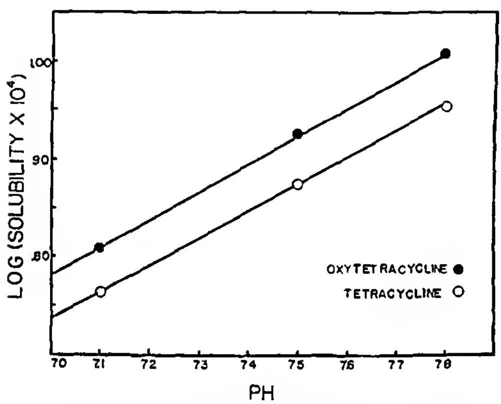

Fig 12 —Plot showing the variation in solubility of tetracycline and oxytetracycline with pH at 25° in the absence of buffer

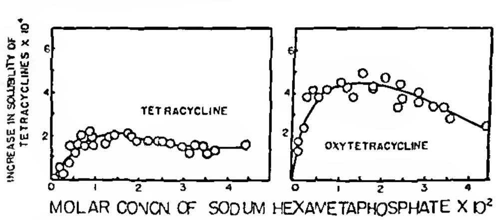

Fig 13 —Plot showing the increase in solubility of the tetracyclines *vs* concentration of sodium hexametaphosphate in the absence of buffer at 25°.

SUMMARY

1. The results of the interaction of a series of homologous aromatic hydroxy, amino, carboxylic acid, and pyridine compounds with oxytetracycline seem to indicate that there exists a relationship between activity and position of functional groups. There also seems to be a correlation between activity and the number and type of polar groups present

2 Aromaticity seems to be essential for optimum complexing activity as determined by experiments with a variety of nonaromatic compounds Also the presence of fused ring systems seems to enhance complexing activity. There is a suggestion of Pi complexing playing an important role in these interactions

3 Oxytetracycline forms complexes with a variety of metabolites, vitamins, and amino acids These interactions suggest a possible mechanism of action *in vivo*

4 A combination of calcium or magnesium ions and various organic complexing agents resulted in a synergistic action as noted by the increase in solubility of oxytetracycline. EDTA decreased the apparent solubility of the oxytetracycline-magnesium chelate probably due to removal of metal ion in a competitive reaction

5 Only little interaction between sodium hexametaphosphate and oxytetracycline and tetracycline was observed. This is not meant to imply that increased oral absorption of this combination does not occur via other physical or chemical pathways

6 At best, only suggestions of the mechanism of action of these interactions can be made due to the complex nature of the systems as well as the presence of inseparable competing reactions

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Isotonic Solutions IX*

The Permeability of Red Corpuscles to Some Monohydric and Polyhydric Alcohols

By PAUL ZANOWIAK† and WILLIAM J. HUSA

The degree of hemolysis of rabbit and human erythrocytes in solutions of a series of monohydric and polyhydric alcohols was investigated. When possible, these data were used to calculate van't Hoff *i* values. The monohydric and polyhydric alcohols which had a molecular weight less than that of erythritol caused complete hemolysis in concentrations calculated to be isosmotic. Certain concentrations of the monohydric alcohols caused hemolysis even in the presence of 0.6 per cent (0.1 *M*) sodium chloride. The low molecular weight diols and triol investigated did not cause hemolysis in the presence of 0.6 per cent sodium chloride. The polyhydric alcohols of higher molecular weight prevented hemolysis. It appeared that the phenomena occurring at the erythrocyte membrane in the case of the alcohols might be influenced by molecular weight and the number of hydroxyl groups present; the ratio of hydroxyl groups to the number of carbon atoms in the molecule might be important.

In an investigation of isotonic solutions of various compounds, Husa and Adams (1) showed that certain of these, including several alcohols, did not prevent hemolysis at concentrations which were calculated to be isotonic according to physicochemical data. Grosicki and Husa (2) stressed the importance of making hemolytic tests, especially as a check on physicochemical data, for the preparation of parenteral solutions. They devised a hemolytic method for determining sodium chloride equivalents and calculated isotonic coefficients for various amino acids, sugars, and salts Husa and co-workers (3-6), employing the hemolytic method, studied the effects of urea and urea derivatives, numerous electrolytes, salts of organic acids, and alkaloidal salts upon rabbit and human red blood cells.

Many organic hydroxy compounds have been found to penetrate erythrocytes of various species However, it has been shown that some sugars did not possess this ability (2). The purpose of this investigation was to determine, wherever possible, the isotonic coefficients of a series of alcohols and to obtain data that might contribute to a hypothesis which could correlate the molecular weight and chemical configuration of alcohols with their erythrocyte membrane activity A hemolytic method was used with rabbit and human red blood cells.

EXPERIMENTAL

Collection of Blood.—The blood samples employed in the present investigation were obtained and treated in the same manner as described previously by Husa and co-workers (2, 3). Human blood samples were obtained mainly from the veins of the arms of a twenty-four year old male. Several experiments were conducted with venous blood of other donors.

Preparation of Solutions.—Stock solutions of various concentrations of the alcohols were prepared by weighing the proper amounts of the respective alcohols and bringing to volume with triple-distilled water. Sodium chloride solutions were prepared in the same way. Necessary dilutions were then made from these stock solutions in the manner described previously (3), as were the solutions containing both sodium chloride and an alcohol

Quantitative Determination of Per Cent Hemolysis.—The method used to determine the degree of hemolysis of erythrocytes in the various solutions involved in this investigation was essentially that of Hunter (7) and has been described in detail by Grosicki and Husa (2). The major difference in procedure in the present investigation was that all experiments were carried out at a temperature of 37° ± 0.1°.

Calculation of *i* Values.—When the concentrations of sodium chloride and any other compound needed to cause a certain degree of hemolysis are known, the isotonic coefficient (*i* value) of the other compound can be calculated according to the osmotic equation used by Grosicki and Husa (2). This was the basis for the *i* value calculations in the present investigation.

In experiments carried out in the presence of 0.2% sodium chloride, the concentration of sodium chloride used in the osmotic equation was obtained by subtracting 0.2 Gm./100 ml. sodium chloride from values determined by interpolation of curves (concentration *vs.* per cent hemolysis) drawn for each blood sample (6).

DISCUSSION

Variability of Blood.—The importance of the variability factor of blood samples used in quantitative hemolytic determinations has been discussed by Husa and co-workers (5, 6). A range of concentration of sodium chloride solutions was employed in this investigation as a standard of reference to cir-

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cumvent error ascribable to the variability of erythrocyte fragility. The degree of hemolysis occurring in the various concentrations of sodium chloride was determined for each blood sample and differences in erythrocyte fragility were overcome by comparison of these values with those found for the degrees of hemolysis in solution of the substance under investigation.

Procedural features that were utilized to diminish experimental error were the use of duplicative tubes of all solutions within each experiment and the use of five separate tubes for 100% hemolysis for each blood sample; three of these tubes were placed at the beginning and two at the end of each run. For each compound studied, at least two experiments, utilizing two different blood samples of the same species, were performed.

Value of *i* for Sodium Chloride.—Previous hemolytic determinations (1–6) were carried out at 25° and the *i* values were calculated using sodium chloride as the standard and 1.86 as the *i* value for sodium chloride. The present investigation was carried out at 37°. It, therefore, became necessary to determine whether the *i* value for sodium chloride would be different at this temperature than at 25° as used by previous workers.

From electromotive and boiling point data, the osmotic coefficient of sodium chloride from 0 to 40° and from 0.1 *M* to 4.0 *M* has been computed and graphically summarized by Harned and Owen (8). For 0.1 *M* sodium chloride there was no significant change of the osmotic coefficient from 0 to 40°, when derived from such data.

It also seemed desirable to obtain hemolytic data which could validate the use of 1.86 as the *i* value for sodium chloride at 37°. Therefore, an experiment was designed employing the general range of sodium chloride concentrations used in conjunction with rabbit blood in this investigation. The procedure was essentially the same as described above except that four tubes of each concentration were used; two each were kept at 25° for forty-five minutes, while two were maintained at 37° for the same period. The tubes held at 25° were regarded as the "known" factor for this experiment and the value for *i* at that temperature was taken as 1.86. From the data so obtained, a value of *i* for sodium chloride at 37° was determined. The results indicated that the *i* values for sodium chloride obtained at 37° were directly comparable with the data obtained at 25° by previous workers and 1.86 was used as the *i* value for sodium chloride in this investigation.

Results with Monohydric Alcohols.—Quantitative hemolytic determinations were performed with methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl and *tert*-butyl alcohols, employing rabbit and/or human blood. The general range of concentration of the solutions of the monohydric compounds was 0.5 to 10% except where the respective solubilities prevented this, in which case the highest practical concentrations were used, e. g., 4% (0.5*M*) of isobutyl alcohol. Other exceptions occurred when after mixing with blood, precipitates formed at the higher concentrations employed, as with *n*-propyl and *n*-butyl alcohols. In these cases the highest practical concentrations were also employed.

In each determination involving the monohydric compounds the highest concentration used was well above that needed to give the same freezing point as a 0.5% solution (0.1 *M*) of sodium chloride. Such a solution might be expected to prevent hemolysis of rabbit and human erythrocytes. However, the results indicated that approximately 100% hemolysis occurred with such concentrations, as well as with the lower concentrations investigated. These data seemed to agree with the results of Griyns (9) who noted that chicken and horse erythrocytes hemolyzed in the presence of aliphatic monohydric alcohols and glycerin. Booij, *et al.* (10), found that normal alcohols at higher concentrations produced hemolysis.

The results obtained from determinations involving certain concentrations of various monohydric alcohols in the presence of 0.2% (0.03 *M*) and 0.6% (0.1 *M*) sodium chloride are summarized in Tables I and II respectively. Table II indicates that all the monohydric alcohols investigated, except methyl alcohol, produced hemolysis in the presence of 0.6% sodium chloride. The effect of a range of concentration of each of these compounds upon human and rabbit erythrocytes in the presence of 0.6% sodium chloride was also investigated.

In the case of ethyl alcohol in the presence of 0.6% sodium chloride, solutions of 5% (1.1 *M*) or less did not cause hemolysis of rabbit and human blood. However, 15% (3.3 *M*) solutions produced complete hemolysis. For *n*-butyl alcohol in the presence of 0.6% sodium chloride, solutions of 1.5% (0.2 *M*) or less did not cause hemolysis of rabbit or human erythrocytes, but 2% solutions (0.3 *M*) produced complete hemolysis.

When rabbit erythrocytes were employed, solu-

TABLE I.—EFFECT OF VARIOUS MONOHYDRIC ALCOHOLS ON HUMAN AND/OR RABBIT ERYTHROCYTES IN THE PRESENCE OF 0.2% SODIUM CHLORIDE

Compound	Rabbit Erythrocytes %	Rabbit Hemolysis, Approximate %	Human Erythrocytes %	Human Hemolysis, Approximate %
Methyl alcohol	..	..	10	100
Ethyl alcohol	10	100	10	100
n-Propyl alcohol	7	100	7	100
Isopropyl alcohol	10	100	..	..
n-Butyl alcohol	2	100	2	100
Isobutyl alcohol	4	100	..	..
sec-Butyl alcohol	5	100	..	..
tert-Butyl alcohol	10	ppt.	..	..

TABLE II.—EFFECT OF VARIOUS MONOHYDRIC ALCOHOLS ON HUMAN AND/OR RABBIT ERYTHROCYTES IN THE PRESENCE OF 0.6% SODIUM CHLORIDE

Compound	Rabbit Erythrocytes %	Rabbit Hemolysis, Approximate %	Human Erythrocytes %	Human Hemolysis, Approximate %
Methyl alcohol	..	..	10	0
Ethyl alcohol	10	70	10	70
n-Propyl alcohol	7	100	7	100
Isopropyl alcohol	10	100	..	..
n-Butyl alcohol	2	100	2	100
Isobutyl alcohol	4	100	..	..
sec-Butyl alcohol	5	100	..	..
tert-Butyl alcohol	10	ppt.	..	..

tions of 2% (0.3 M) or less, of n-propyl alcohol in the presence of 0.6% sodium chloride did not cause hemolysis, but 5% solutions (0.8 M) produced complete hemolysis. With human erythrocytes, 4% solution (0.7 M) or less did not cause hemolysis, while 5% (0.8 M) solution produced complete hemolysis.

In the presence of 0.6% sodium chloride, solutions of 5% (0.8 M) of isopropyl alcohol did not cause hemolysis of rabbit erythrocytes, but solutions of 10% (1.7 M) produced complete hemolysis. Solutions of isobutyl alcohol of 1.5% (0.2 M) or less in the presence of 0.6% sodium chloride did not cause hemolysis; however, 2% solutions (0.3 M) caused approximately 60% hemolysis. Solutions of 2% (0.3 M) of *sec*-butyl alcohol in the presence of 0.6% sodium chloride caused approximately 5% hemolysis of rabbit blood, while 4 and 5% solutions (0.5 M and 0.7 M) caused complete hemolysis. Similarly, 5% solutions (0.7 M) of *tert*-butyl alcohol in the presence of 0.6% sodium chloride caused approximately 5% hemolysis, whereas 7% solutions (0.9 M) caused complete hemolysis.

In the case of the monohydric alcohols which were found to produce hemolysis in the presence of 0.6% sodium chloride, the degree of hemolysis seemed to increase with an increase in the concentration of the respective compounds. For each of these compounds, as outlined in the above discussion, there appeared to be a certain critical concentration below which, in the presence of 0.6% sodium chloride, hemolysis did not occur.

At certain higher concentrations of these alcohols, precipitates formed when 0.6% sodium chloride was present. This precipitation may have been due to denaturation of one or more of the proteins of the blood.

Results with Certain Polyhydric Alcohols.—Quantitative determinations were carried out employing ethylene glycol, 1,2-propanediol, glycerol, and 1,3-butanediol in concentrations of 0.5 to 10%. In each determination involving these compounds, the 10% solution was well above the concentration needed, according to osmotic calculations, to have the same osmotic pressure as a 0.5% (0.1 M) solution of sodium chloride. The results indicated that approximately 100% hemolysis occurred with this concentration, as well as with lower concentrations investigated

Abnormal permeability of erythrocytes in solutions of some nonelectrolytes can be corrected in some cases by the addition of a small proportion of an electrolyte such as sodium chloride (2). The effect of the presence of 0.2% (0.03 M) and 0.6% (0.1 M) sodium chloride upon the hemolysis by ethylene glycol, 1,2-propanediol, glycerol, and 1,3-butanediol has been studied in the present investigation. In each case, 10% solutions of the respective compounds were used. Regardless of the type of erythrocytes employed, all of these solutions caused complete hemolysis in the presence of 0.2% sodium chloride. In the presence of 0.6% sodium chloride the 10% solutions [(1.1 M) to (1.6 M)] of each of these compounds did not cause hemolysis.

Results with Other Polyhydric Alcohols.—It was possible to determine i values for certain of the polyhydric alcohols investigated. These compounds and the average i values found for each, are given in Tables III and IV which also include their i values determined in the presence of 0.2% (0.03 M) sodium chloride. These data seem in agreement with several investigations reported in the literature. Griyns (9) found that chicken and horse erythrocytes remained intact in isotonic solutions of mannitol and inositol. Hedin (11) reported that mannitol and adonitol penetrated ox erythrocytes only slightly and erythritol somewhat more rapidly. Human erythrocytes were found to be impermeable to mannitol and dulcitol and slightly permeable to adonitol (12). Five and six carbon alcohols did not perceptibly penetrate human erythrocytes (13).

TABLE III.—VALUES FOR i FOR VARIOUS POLYHYDRIC ALCOHOLS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

Compound	Hemolysis, % 25	50	75	Average
Mannitol	1.26	1.29	1.33	1.29
Sorbitol	1.23	1.28	1.33	1.28
Inositol[b]	1.27	1.30	1.35	1.31
Mannitol[c]	1.23	1.24	1.26	1.24
Sorbitol[c]	1.22	1.24	1.28	1.25
Inositol[c]	1.18	1.22	1.26	1.22

[a] Unless otherwise indicated, all i values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In the presence of 0.2% sodium chloride.

TABLE IV.—VALUES FOR i FOR VARIOUS POLYHYDRIC ALCOHOLS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

Compound	Hemolysis, % 25	50	75	Average
Erythritol	0.47	0.53	0.60	0.53
Pentaerythritol	0.61	0.66	0.72	0.66
Mannitol	1.32	1.37	1.42	1.37
Sorbitol	1.31	1.37	1.41	1.36
Inositol[b]	1.35	1.40	1.46	1.40
Mannitol[c]	1.20	1.21	1.25	1.22
Sorbitol[c]	1.19	1.21	1.24	1.21
Inositol[c]	1.20	1.24	1.30	1.25

[a] Unless otherwise indicated, all i values represent an average of two blood samples
[b] Average of three blood samples.
[c] In presence of 0.2% sodium chloride.

Values for i of approximately 1.20 for amino acids and sugars using rabbit erythrocytes were reported by Grosicki and Husa (2). They found similar i values for dextrose, sucrose, and lactose, also with rabbit erythrocytes. Reference to Tables III and IV shows i values for mannitol, sorbitol, and inositol to be approximately 1.40 with human erythrocytes and 1.30 with rabbit erythrocytes. These values might be interpreted as showing 30 to 40% dissociation of these polyhydric alcohols, but this would be contrary to the recognized chemical properties of such compounds. In solutions of some substances erythrocytes lose electrolytes from within the cell, thus causing a decrease in the internal osmotic pressure and a corresponding increase in the resistance to hemolysis. Grosicki and Husa (2) hypothesized that such exosmosis from erythrocytes might have occurred in the solutions of amino acids and sugars they studied Electrolyte loss would cause the cells to be in equilibrium with lower concentrations of the amino acids and sugars than would otherwise be the case, and the i values would be higher than if no

electrolyte loss had occurred Thus, the fact that the i values for mannitol, sorbitol, and inositol were found to be greater than 1 00 might be ascribed to loss of electrolytes from within the erythrocytes

Hober (14) stated that the severe alteration of the normal permeability of erythrocytes caused by isotonic solutions of some nonelectrolytes could be largely reduced by the addition of small proportions of an electrolyte such as sodium chloride Reference to Tables III and IV reveals the effects of 0 2% sodium chloride upon the i values of mannitol, sorbitol, and inositol in rabbit and human blood Using rabbit erythrocytes there appeared to be little difference in the i values determined in the presence of 0 2% sodium chloride and the i values determined with the alcohols alone With human erythrocytes the i values seem to be slightly lower in the presence of 0 2% sodium chloride However, even after the addition of 0 2% sodium chloride, these i values are still greater than 1 00 This situation is similar to that reported by Grosicki and Husa (2) for the sugars they investigated using rabbit erythrocytes, even in the presence of 0 2% sodium chloride the i values of the sugars remained approximately 1 20 They stated that the presence of sodium chloride apparently did not correct the abnormal exosmosis With mannitol, sorbitol, and inositol the presence of of 0 2% sodium chloride apparently also did not alter abnormal exosmosis

Using human erythrocytes, Grosicki and Husa (2) reported an average i value of 1 17 for dextrose In the presence of 0 2% sodium chloride, dextrose gave an i value of 0 66 In the present investigation pentaerythritol alone was found to have an average i value of 0 66 and erythritol 0 53, when human erythrocytes were employed Such low i values might indicate a degree of permeability of the erythrocytes by the compounds in question

Structure and Activity.—The monohydric alcohols investigated which contained from one to four carbon atoms did not prevent hemolysis The difficulty in clearly relating hemolytic effects and chemical structure of the straight chain alcohols was noted by Ponder (15) Fuhner and Neubauer (16) reported that the hemolytic action of alcohols increased with increasing molecular weight Ponder and Hyman (17) found that primary alcohols of three to nine carbon atoms showed a logarithmic relationship between the number of carbon atoms and the power to accelerate the hemolytic effect of saponin solutions Alcohols containing three or less carbon atoms were reported to rapidly hemolyze dog erythrocytes (18)

Various isomerically related monohydric alcohols were included in the present investigation There appeared to be little difference between the hemolytic effects of n propyl and isopropyl alcohols and n-butyl, isobutyl, *sec* butyl, and *tert* butyl alcohols even in the presence of sodium chloride However, branch chain alcohols were reported by Seelich and Pirquit (19), to possess weaker hemolytic effects than the corresponding normal alcohols, the results in Table II are in accord with this statement, since n-butyl alcohol in the presence of 0 6% sodium chloride caused 100% hemolysis at a lower concentration than the branched chain isomers

Several polyhydric alcohols (ethylene glycol, 1,2-propanediol, glycerol, and 1,3 butanediol) did not show the ability to prevent hemolysis Furthermore, in the presence of 0 2% sodium chloride no preventive tendencies were found However, in the presence of 0 6% sodium chloride no hemolysis was noted

Jacobs and co workers (20) found that each successive hydroxyl group added to the propane molecule decreased the rate of penetration of ox and rabbit erythrocytes In the diols, the position of the hydroxyl groups seemed important, for penetration occurred more rapidly when these groups were on adjacent carbon atoms than when they were located at the ends of the molecule In the present investigation, there seemed to be no difference in the hemolytic activities of n propyl alcohol, isopropyl alcohol, 1,2 propanediol, and glycerol when these were used alone or in the presence of 0 2% sodium chloride However, when 0 6% sodium chloride was employed, glycerol and 1,2 propanediol did not cause hemolysis, whereas n propyl and isopropyl alcohols caused hemolysis A similar situation was found in the case of the four carbon alcohols investigated None of the isomeric butyl alcohols nor 1,3 butanediol prevented hemolysis when used alone or in the presence of 0 2% sodium chloride When 0 6% sodium chloride was used, 1,3 butanediol did not cause hemolysis, whereas the isomeric butyl alcohols did cause hemolysis Erythritol, furthermore, was found to prevent hemolysis, having an i value of approximately 0 55

It was possible to obtain i values for the five carbon alcohol, pentaerythritol, and the six carbon alcohols, sorbitol, mannitol, and inositol, indicating that these compounds were able to prevent hemolysis Hedin (11) observed that the rate of penetration of polyhydric alcohols decreased with the number of hydroxyl groups present Erythritol was reported (18) as causing a slower hemolysis of dog erythrocytes than alcohols of three or less carbon atoms, five- and six carbon alcohols were found to hemolyze the blood samples very slowly

SUMMARY

1 The degree of hemolysis of rabbit and human erythrocytes in solutions of monohydric and polyhydric alcohols of varying concentration was determined quantitatively and compared with that occurring in various concentrations of sodium chloride When possible, these data were used to calculate van't Hoff i values

2 Approximately 100 per cent hemolysis occurred, at concentrations calculated to be isosmotic, with all of the alcohols investigated which had a molecular weight less than that of erythritol, those of greater molecular weight prevented hemolysis

3 The alcohols of lower molecular weight were found also to cause complete hemolysis in the presence of 0 2 per cent sodium chloride Of these, the monohydric alcohols, when present in lower concentrations, did not cause hemolysis in the presence of 0 6 per cent sodium chloride, in higher concentrations, however, except for methyl alcohol, the monohydric alcohols caused

hemolysis in the presence of 0.6 per cent sodium chloride This was true of the normal monohydric alcohols investigated as well as their isomers

4 The low molecular weight diols and triol investigated did not cause hemolysis in the presence of 0.6 per cent sodium chloride.

5. For erythritol and pentaerythritol *i* values of less than 1.00 were found, possibly indicating a degree of permeability of the erythrocytes by these compounds.

6. Mannitol, sorbitol, and inositol showed *i* values of approximately 1.40 with human blood and 1 30 with rabbit blood. Values for *i* which are greater than 1.00 might be ascribed to a loss of electrolytes from within the erythrocytes.

7. It appeared that the phenomena occurring at the erythrocyte membrane in the case of alcohols might be influenced by molecular weight and the number of hydroxyl groups present. When an increase in molecular weight was due to hydroxyl groups, the degree of hemolysis occurring seemed to decrease. Polyhydric compounds of four or more hydroxyl groups and four or more carbon atoms prevented hemolysis. Thus, the ratio of hydroxyl groups to carbon atoms present in the molecule might be important

8 The results indicated that the *i* values for sodium chloride obtained at 37° were directly comparable with the data obtained at 25° by previous workers.

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Isotonic Solutions X*

The Permeability of Red Corpuscles to Various Local Anesthetics

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In the present investigation, the effects of local anesthetics in preventing hemolysis of human and rabbit erythrocytes were studied. Employing the hemolytic method, van't Hoff *i* values were obtained for several local anesthetics. The results indicate that these compounds have lower *i* values than might be expected. The addition of 0.2 per cent sodium chloride depressed the *i* values. Hemolytic *i* values were not determinable for the majority of the compounds studied; these compounds caused hemolysis even in the presence of 0.6 per cent sodium chloride.

THE ADVANTAGE of applying the hemolytic method to the preparation of isotonic solutions was pointed out by the investigations of Husa and co-workers (1-8) They emphasized that the osmotic effect of a substance upon an erythrocyte membrane depends not only upon the number of particles contributed by the solute but also upon whether or not they penetrate the membrane or liberate hemoglobin in some other manner If the compound under consideration does not exhibit the *i* value expected on the basis of the number of ions it is theoretically presumed to contribute, then extra deliberation is indicated in the preparation of isotonic solutions of the substance These observations make it apparent that calculation of tonicity, based solely upon colligative properties, may prove to be inaccurate

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when applied to the preparation of solutions isotonic with the blood.

In the present investigation, an inquiry was made into the effects of local anesthetics in preventing hemolysis of human and rabbit blood. An examination of the literature revealed only two references (9, 10) relating to the effect of local anesthetics upon the red corpuscle; the majority of the compounds studied in this investigation had not been previously reported with reference to their effect upon erythrocytes. The hemolytic *i* values for several anesthetics were determined. Experiments were also conducted to determine the effect that the local anesthetics have in preventing hemolysis when used in the presence of 0.2 per cent sodium chloride. It has been previously reported (11) that the addition of small proportions of an electrolyte, such as sodium chloride, reduces the severe alteration of permeability that occurs when erythrocytes are placed in solutions of some nonelectrolytes. Hartman and Husa (4) observed that this is also true in solutions of some electrolytes. Determinations were similarly conducted in the presence of 0.6 per cent sodium chloride; the purpose of these experiments was to evaluate the effect of these compounds upon red corpuscles even when the salt concentration was sufficient to prevent osmotic hemolysis if no other substance were present.

EXPERIMENTAL

Collection of Blood.—The blood used in the present investigation was obtained in the same manner as described previously by Husa and coworkers (2, 3). The human blood samples were obtained from the veins of the arms of a twenty-seven-year-old male. Several experiments were carried out employing blood from other donors.

Preparation of Solutions.—The solutions used in this study were prepared in essentially the same manner as described by other workers (2, 3).

Quantitative Determination of Per Cent Hemolysis.—The calculation of hemolytic *i* values has been described in detail by Grosicki and Husa and Easterly and Husa (2, 3).

DISCUSSION

Variability of Blood.—A range of concentrations of sodium chloride solutions was employed for each blood sample. Thus, any differences in fragility were compensated for by comparison of the degree of hemolysis occurring in solutions of the substance under investigation with the degree of hemolysis in various concentrations of sodium chloride. Duplicate tubes of all solutions were employed within each experiment. Five separate tubes were used for each blood sample for the 100% hemolysis readings; two tubes were placed at the beginning and three at the end of the experimental run. For each local anesthetic studied experiments were conducted using at least two different blood samples of the same species.

Chemicals.—Samples of the local anesthetics employed in this investigation were generously contributed by their manufacturers: butacaine sulfate, procaine hydrochloride, and pramoxine hydrochloride by Abbott Laboratories; lidocaine hydrochloride by Astra Pharmaceutical Products, Inc.; dibucaine hydrochloride by Ciba Pharmaceutical Products, Inc.; cyclomethycaine hydrochloride and piperocaine hydrochloride by Eli Lilly and Co.; diperodon hydrochloride by the Wm. S. Merrell Co.; hexylcaine hydrochloride by Merck Sharp & Dohme Research Laboratories; intracaine hydrochloride by Philadelphia Ampoule Labs.; procaine isobutyrate by William H. Rorer, Inc.; benoxinate hydrochloride by Smith-Dorsey; procaine amide hydrochloride by E. R. Squibb & Sons; 2-chloroprocaine hydrochloride by Wallace & Tiernan Inc.; phenacaine hydrochloride, 2-propoxyprocaine hydrochloride, and tetracaine hydrochloride by Sterling-Winthrop Research Institute.

Results with Procaine Hydrochloride, Procaine Isobutyrate, and Procaine Amide Hydrochloride.—Procaine hydrochloride apparently exhibited some osmotic effect in preventing hemolysis in human and rabbit blood. Hemolytic *i* values were determinable only for 75% hemolysis of human blood and for 50% and 75% hemolysis of rabbit blood. There are two opposing variables. The first is the decrease in hemolysis due to an increase in the number of particles in solution (increasing concentration). The second is the increase in hemolysis due to the effect of increasing concentration of the compound upon the corpuscular membrane. The curve for per cent hemolysis *versus* concentration is therefore the composite curve for these two variables. There were several instances where hemolytic *i* values were only obtainable from 75% or 50% and 75% hemolysis values (see Tables I and II). This was due to the effect of the anesthetic upon the red cell membrane in preventing the development of a normal sigmoid curve, i.e., a curve from which 25, 50, and 75% hemolysis values are obtainable. Procaine hydrochloride solutions caused a significant proportion of hemolysis in the presence of 0.6% sodium chloride. Thus the local anesthetic apparently had an appreciable effect upon the corpuscular membrane. The average hemolytic *i* value for procaine hydrochloride, in human blood at 75% hemolysis was 0.57; in rabbit blood, at 50% and 75% hemolysis, the *i* value was 0.49.

The presence of 0.2% sodium chloride has been reported to restore the normal permeability of erythrocytes in some instances (2, 4, 6, 11). The average value of *i* for procaine hydrochloride, in the presence of 0.2% sodium chloride, was obtainable only for 75% hemolysis of both human and rabbit blood; these values were 0.30 and 0.28, respectively. It has been reported that the addition of sodium chloride to a solution of a nitrogenous basic hydrochloride represses the dissociation of the nitrogenous base so markedly that it behaves almost like a nonelectrolyte (12). The degree of repression of dissociation is determined by the basic strength of the nitrogenous base Thus the number of osmotically active particles is dependent upon the concentra-

TABLE I.—VALUES OF i FOR VARIOUS LOCAL ANESTHETICS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

Anesthetic Hydrochloride	Hemolysis, % 25	50	75	Average
Procaine[b]	...	...	0.57	0.57
Procaine[c]	...	...	0.30	0.30
Procaine amide[b]	0.37	0.44	0.50	0.43
Procaine amide[b, c]	. .	0.27	0.28	0.28
2-Propoxyprocaine	0.81	1.27	1.67	1.25
2-Chloroprocaine	...	...	1.42	1.42
2-Chloroprocaine[c]	. .	...	0.77	0.77

[a] Unless otherwise indicated all i values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In presence of 0.2% sodium chloride.

TABLE II.—VALUES OF i FOR VARIOUS LOCAL ANESTHETICS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

Anesthetic Hydrochloride	Hemolysis, % 25	50	75	Average
Procaine[b]	...	0.46	0.52	0.49
Procaine[c]	...	...	0.28	0.28
Procaine amide[b]	...	...	0.42	0.42
Procaine amide[c]	...	. .	0.26	0.26
2-Propoxyprocaine	0.79	0.94	1.12	0.95
2-Chloroprocaine	...	...	0 68	0.68
2-Chloroprocaine[c]	...	...	0.31	0.31

[a] Unless otherwise indicated all i values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In presence of 0.2% sodium chloride.

tion of the basic hydrochloride and sodium chloride as well as the character of the basic hydrochloride (12). The lower i values obtained in the presence of sodium chloride might be ascribed to the repression of the dissociation of procaine hydrochloride by the salt. Thomasson (6) investigated the effect of 0.2% sodium chloride upon the hemolytic i values of various alkaloids. He found that the addition of sodium chloride lowered the i values of atropine sulfate, ephedrine hydrochloride and sulfate, and homatropine methylbromide.

Procaine isobutyrate failed to show any appreciable prevention of hemolysis at concentrations that were calculated to be isosmotic. The difference in permeability between procaine hydrochloride and procaine isobutyrate may be explained on the basis of the pH of these compounds. A 1% solution of the hydrochloride had a pH of 5.23 whereas the isobutyrate had a pH of 7.05. Goyan and Daniels (13) presented the equilibria involved in a solution of a procaine salt and stated that in general, neutral or slightly alkaline solutions favor a high equilibrium concentration of the free base. There have been many references to substantiate the premise that the free bases of weak electrolytes are able to penetrate the cell membrane while the cation cannot (14–17). As a consequence, it is to be expected that the isobutyrate would more easily penetrate the red cell than the hydrochloride due to the higher concentration of the free base, i. e., procaine isobutyrate would exhibit little or no appreciable osmotic effect.

The average hemolytic i value for procaine amide in human blood was 0.43 and in rabbit blood, at 75% hemolysis, was 0.42. The determinations involving human blood provided characteristically sigmoid curves; however, the per cent hemolysis could not be brought to zero. Instead, the curve indicated increased hemolysis at 10% (0.37 M) concentration of anesthetic. A possible explanation for this phenomenon would be the increased fragility of the erythrocytes noted at 10% concentration of anesthetic in the presence of 0.6% sodium chloride. Since hemolysis occurred to some extent throughout the range of concentrations employed, the low i values could be attributed to this cause, i.e., the hemolysis due to the alteration of the membrane would release hemoglobin in addition to that already liberated from osmotic hemolysis. Rabbit cells were more sensitive to procaine amide and thus 25% and 50% hemolysis values were unobtainable.

The addition of 0.2% sodium chloride to procaine amide solutions lowered the hemolytic i values to 0.28 in human blood and to 0.26 in rabbit blood.

Results With 2-Propoxyprocaine, 2-Chloroprocaine, and Benoxinate Hydrochlorides.—The hemolytic i values for 2-propoxyprocaine hydrochloride were 1.25 in human blood and 0.95 in rabbit blood. Determinations were carried out in the presence of 0.6% sodium chloride and no significant degree of hemolysis was noted at the concentrations utilized in the calculation of hemolytic i values.

The hemolytic i values for 2-chloroprocaine hydrochloride were obtainable only for 75% hemolysis; the average value in human blood was 1.42, for rabbit blood 0.68. The addition of 0.2% sodium chloride reduced these values to 0.77 and 0.31, respectively. At the concentrations employed in the calculations of the i values, in both human and rabbit blood, approximately 50% hemolysis was observed in the presence of 0.6% sodium chloride. These results indicate that 2-chloroprocaine had an appreciable effect upon the erythrocyte membrane. Bodansky (18) reported that the halogen derivatives of fatty acids were much more lytic than the acids themselves, probably because they were much more highly dissociated and more soluble in lipids. Hober (19) stated that the introduction of polar groups decreased the penetrating capacity of a substance, whereas that of nonpolar groups, such as halogens, increased the penetrating capacity. The data obtained for 2-chloroprocaine apparently does not concur with the aforementioned contention that halogen substitution increases the penetrating capacity of a compound; the hemolytic i values for 2-chloroprocaine were higher than those of procaine. Paradoxically, however, hemolysis occurred at a lower concentration in the presence of 0.6% sodium chloride in solutions of the chlorine-substituted compound (see Table III). This evidence suggests that while the halogenated procaine does not penetrate the erythrocyte as readily as does procaine, it has a more profound effect upon the membrane.

Experiments with benoxinate hydrochloride demonstrated that this compound had no apparent effect in preventing hemolysis. A concentration of 0.42% (1.2×10^{-2} M) benoxinate hydrochloride caused 50% hemolysis of human blood in the presence of 0.6% sodium chloride; in rabbit blood, the concentration was 0.16% (4.6×10^{-3} M). It is of interest to note that 2-propoxyprocaine and benoxinate differ in that the latter is a 3-n-butoxypro-

caine. It seems as if the substitution of the propoxy group with a butoxy group is responsible for the vastly greater hemolytic effect of benoxinate hydrochloride.

A partial explanation for the unusually low hemolytic *i* values obtained for procaine, procaine amide, 2-propoxyprocaine, and 2-chloroprocaine hydrochlorides may lie in a consideration of the possible formation of micellular aggregates by these compounds Debye (20, 21) reported that substituted quaternary ammonium salts and amine hydrochlorides often form micellular aggregates in solution. Hammarlund and Pedersen-Bjergaard (22) indicated that solutions of dibucaine (0 07 *M*), tetracaine (0 13 *M*), and pramoxine (0 08 *M*) hydrochlorides behaved in a manner suggesting the formation of micelles. The formation of such aggregates would produce fewer particles in solution than expected from the original concentration and would consequently result in lower *i* values The addition of any substance which would disturb the micellular formation by either increasing or decreasing the number of particles would alter the osmotic effect; 0 2% sodium chloride might act in such a manner. The formation of micelles by these compounds has not been experimentally established; however, the substituted procaine compounds are structurally similar to tetracaine, are amine hydrochlorides and would therefore be expected to behave similarly to some extent In any event, this concept would provide only a partial explanation for the low *i* values; the data from hemolytic determinations in 0 6% sodium chloride indicated that these anesthetics caused some hemolysis regardless of osmotic pressure.

Results with Esters of Benzoic and Para-aminobenzoic Acid in the Presence of 0.6% Sodium Chloride.—The anesthetics of this group that were investigated were cyclomethycaine, hexylcaine, intracaine, piperocaine, and tetracaine hydrochlorides, and butacaine sulfate. Preliminary hemolytic determinations disclosed that these compounds had no apparent effect in inhibiting hemolysis in concentrations that were supposedly isotonic according to physicochemical data In compounds of this general structure, the alteration or addition of groups may produce a profound effect upon their properties as regards hemolysis For example, piperocaine and cyclomethycaine differ only in the presence of a *p*-cyclohexyloxy group on the benzoic acid moiety of the latter compound. The addition of this substituent group is apparently responsible for the vastly greater hemolytic properties of cyclomethycaine. The concentrations of local anesthetic causing 50% hemolysis of erythrocytes in 0.6% sodium chloride is presented in Tables III and IV In human blood, the hemolytic activity increased in the following order: intracaine, piperocaine, hexylcaine, tetracaine, butacaine, and cyclomethycaine. In rabbit blood the order was the same with the exception that hexylcaine precedes piperocaine. Cyclomethycaine caused hemolysis at the lowest concentration of all the compounds examined

Results with Anesthetics of Heterogeneous Structure in the Presence of 0.6% Sodium Chloride.—A study was made of diethylaminoethanol, dimethylaminoethanol, and dibucaine, diperodon, lidocaine, phenacaine, and pramoxine hydrochlorides.

TABLE III.—CONCENTRATIONS OF LOCAL ANESTHETIC CAUSING 50% HEMOLYSIS OF HUMAN ERYTHROCYTES IN THE PRESENCE OF 0.6% SODIUM CHLORIDE[a]

Local Anesthetic[b]	Molarity	Per Cent
Procaine amide	[c]	...
2-Propoxyprocaine	[c]	..
Procaine	$3\ 1 \times 10^{-1}$	8 6
Procaine isobutyrate	$2\ 0 \times 10^{-1}$	6 9
2-Chloroprocaine	$1\ 6 \times 10^{-1}$	5 1
Lidocaine	$1\ 5 \times 10^{-1}$	4 2
Intracaine	$1\ 1 \times 10^{-1}$	3.2
Piperocaine	$8\ 5 \times 10^{-2}$	2 5
Hexylcaine	$2\ 9 \times 10^{-2}$	0 87
Phenacaine	$1\ 4 \times 10^{-2}$	0 49
Benoxinate	$1\ 2 \times 10^{-2}$	0 42
Dimethylaminoethanol[d]	$1\ 1 \times 10^{-2}$	0 10
Tetracaine	$9\ 2 \times 10^{-3}$	0 28
Pramoxine	$6\ 1 \times 10^{-3}$	0.2
Butacaine sulfate	$5\ 6 \times 10^{-3}$	0 40
Diethylaminoethanol[d]	$4\ 1 \times 10^{-3}$	0 05
Diperodon	$2\ 6 \times 10^{-3}$	0 11
Dibucaine	$1\ 8 \times 10^{-3}$	0 07
Cyclomethycaine	$5\ 5 \times 10^{-4}$	0 02

[a] Values obtained by interpolation from curves of per cent hemolysis *versus* concentration, values represent average of two blood samples
[b] Anesthetic as the hydrochloride unless otherwise indicated
[c] These compounds did not cause 50% hemolysis.
[d] As the alcohol.

TABLE IV.—CONCENTRATION OF LOCAL ANESTHETIC CAUSING 50% HEMOLYSIS OF RABBIT ERYTHROCYTES IN THE PRESENCE OF 0.6% SODIUM CHLORIDE[a]

Local Anesthetic[b]	Molarity	Per Cent
Procaine	[c]	
Intracaine	[c]	
Procaine isobutyrate	$8\ 5 \times 10^{-2}$	2 8
Procaine amide	$8\ 1 \times 10^{-2}$	2 2
2-Propoxyprocaine	$3\ 9 \times 10^{-2}$	1 3
2-Chloroprocaine	$2\ 7 \times 10^{-2}$	0 83
Pramoxine	$2\ 1 \times 10^{-2}$	0 69
Lidocaine	$2\ 0 \times 10^{-2}$	0 53
Hexylcaine	$1\ 7 \times 10^{-2}$	0 52
Piperocaine	$9\ 9 \times 10^{-3}$	0 29
Phenacaine	$8\ 8 \times 10^{-3}$	0 31
Benoxinate	$4\ 6 \times 10^{-3}$	0 16
Tetracaine	$4\ 4 \times 10^{-3}$	0 13
Dimethylaminoethanol[d]	$3\ 6 \times 10^{-3}$	0 03
Butacaine Sulfate	$3\ 4 \times 10^{-3}$	0 24
Diperodon	$3\ 3 \times 10^{-3}$	0 14
Diethylaminoethanol[d]	$1\ 8 \times 10^{-3}$	0 02
Dibucaine	$8\ 9 \times 10^{-4}$	0 034
Cyclomethycaine	$3\ 8 \times 10^{-4}$	0 015

[a] Values obtained by interpolation from curves of per cent hemolysis *versus* concentration, values represent average of two blood samples
[b] Anesthetic as the hydrochloride unless otherwise indicated
[c] These compounds did not cause 50% hemolysis
[d] As the alcohol.

These compounds also did not prevent osmotic hemolysis in concentrations that were presumably isotonic. Several investigators (23, 24) have reported that procaine was hydrolyzed by serum to diethylaminoethanol and *p*-aminobenzoic acid. Dimethylaminoethanol would be the amino alcohol resulting from the hydrolysis of tetracaine. The pH of these two amino alcohols was 10.1 at the concentration causing 50% hemolysis in the presence of 0.6% sodium chloride (see Tables III and IV). The

excessive alkalinity is apparently responsible for the hemolytic effect of these compounds.

The concentrations of dibucaine, diperodon, lidocaine, phenacaine, and pramoxine hydrochlorides causing 50% hemolysis in the presence of sodium chloride are listed in Tables III and IV. Hemolytic effect increased in the following order: lidocaine, phenacaine, pramoxine, diperodon, and dibucaine; in rabbit blood the order was pramoxine, lidocaine, phenacaine, diperodon, and dibucaine

The effect of micelle formation by dibucaine, tetracaine, and pramoxine hydrochlorides, described by Hammarlund and Pedersen-Bjergaard (22), was not reflected in the data obtained in this investigation The explanation for this would seem to lie in the fact that the concentrations of anesthetic used in the hemolytic determinations were considerably lower than those described as forming micellular aggregates

Penetration of the Free Base.—Jacobs (25, 26) made the first analysis of the behavior of the ammonium salts of weak acids. In this case, both anion and cation can penetrate in the unionized form Jacobs came to the conclusion that NH_3, formed by the hydrolysis, is the form in which ammonia penetrates the cell membrane and found that in the case of red cells, hemolysis occurred with NH_4 salts of even strong acids This is because red cells are normally permeable to anions, so that in the case of NH_4Cl, ammonia penetrates as NH_3 and the chloride as the ion, so that there is no impermeable substance to prevent the red cells from swelling and bursting. If the local anesthetics may be considered as $(R_3NH)Cl$, it is possible to make an analogy with NH_4Cl The free base R_3N may then be considered to enter the cell as does NH_3 Husa and Adams (1) found that NH_4Cl was hemolytic even in the presence of 0 6% sodium chloride They concluded that osmotic pressure considerations alone appear insufficient to explain these results; apparently NH_4Cl brings about the release of hemoglobin by some other mechanism, perhaps by dissolving some membrane constituent and thus altering its permeability This explanation may in part explain the hemolytic effects of many of the local anesthetics employed in this investigation

Effect of Cholinesterase Activity.—Several investigators have established that a relationship exists between the permeability of erythrocytes and cholinesterase activity (27, 28, 29) The consensus seems to be that the permeability of red cells would be altered by inhibition of cholinesterase in the cell membrane It has also been confirmed that local anesthetics are inhibitory to cholinesterase; indeed procainesterase was found to be identical with cholinesterase (30–36). A logical assumption would be that the local anesthetics influence erythrocyte permeability to some extent by the inactivation of cholinesterase Alteration of the cell membrane by inhibition of membrane cholinesterase may explain the release of hemoglobin from red corpuscles in solutions of local anesthetics containing 0 6% sodium chloride

SUMMARY

1. Hemolytic *i* values have been determined for several local anesthetics but were not determinable for the majority of the compounds investigated.

2. It was found that the addition of 0 2% sodium chloride lowered the *i* values

3. Most of the local anesthetics studied caused hemolysis even in the presence of 0 6% sodium chloride.

4. In compounds of the benzoic and *p*-aminobenzoic ester type, the alteration or addition of groups produced a profound effect upon their properties as regards hemolysis

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A Study of Permeability to Water Vapor of Fats, Waxes, and Other Enteric Coating Materials*

By TAKERU HIGUCHI and ARMANDO AGUIAR†

Apparent diffusivity and permeability of a variety of materials commonly employed to delay drug release from certain dosage forms have been evaluated using the lag time method. Several cellulose and glyceryl esters were studied and their permeability shown to vary with the relative polarity of these compounds. The influences of such variables as temperature, relative humidity, and compositions of mixtures were investigated. Thermal coefficients obtained for glyceryl monostearate suggest that the apparent overall heat of activation for permeation is about 8 Kcal./mole. The same system exhibits a very high dependency on relative humidity showing proportionally higher permeability near saturation than at lower values.

INFORMATION relative to resistance toward permeation of various packaging and coating substances by aqueous vapor is of considerable importance to those concerned with formulation of pharmaceutical dosages. Since moisture pick up or loss from drug preparations while in storage or on store shelves can often lead to undesirable results, materials highly resistant to moisture permeation are usually employed in pharmaceutical packaging. Many recent container materials, such as some plastics, exhibit rather low impedance to movement of water vapor. *Modus operandi* of some enteric and delayed action dosage forms, on the other hand, appears to depend in part on controlled moisture penetration in regulating the rate of release of active medicaments.

Results of investigation of factors which influence the relative resistance of various plastics and coating materials toward penetration by water vapor are presented in this report. Although some new data on permeation of some plastics have been obtained, the primary aim of this communication is to present new information relative to the permeability of various coating and enteric materials used with or over compressed tablets and related dosage forms to produce delayed or sustained action effect. The influences of composition, temperature, and relative humidity on the apparent diffusivity of and permeability to water molecules have been investigated.

LAWS GOVERNING PERMEATION PROCESS

Based on an analogy of heat conduction through solid media, Fick, in 1855 was able to propose a general law for diffusion. The essence of Fick's law is that the driving force which causes the transfer of a substance from regions of high to regions of low concentration is proportional to the concentration gradient. Fick's law (1) is commonly written as

$$Q = -D\frac{dc}{dx} \qquad \text{(Eq. 1)}$$

where Q is the flux or the amount of material crossing a plane of unit area per unit time, dc/dx the concentration gradient, D is the diffusion coefficient and has the dimensions of cm^2 sec^{-1}. In this work Q has been expressed as cc. of gas at normal temperature and pressure. The negative sign in Fick's law indicates that flow is directed toward the area of decreasing concentration.

The diffusion coefficient is a measure of the amount of material which would diffuse across a unit area under a unit concentration gradient in unit time. The application (2) of Fick's first law for diffusion requires the determination of the flux Q and the concentration gradient. The fact that the law specifies conditions of a constant concentration gradient implies the establishment of a steady state.

The assumption that D is independent of the concentration is not always valid. For exact analysis it becomes necessary to treat D as a variable in deriving an expression for the change of concentration with time. Treating D as a variable, Fick's second law can be written as

$$\frac{dc}{dt} = \frac{d}{dx}\left(D\frac{dc}{dx}\right) \qquad \text{(Eq. 2)}$$

The concentration of the diffusing substance within the surface of a membrane is not always given by the gas or the vapor pressure of the substance, for equilibrium at the interface membrane gas will not be established in such cases where the transition of the gas through the membrane is not a rapid process compared with the diffusion. In such cases an absolute determination of the diffusion constant is not possible and one makes use, therefore, of the concept of a "quasi stationary state." In the quasi stationary state neither the concentrations themselves nor the concentration gradient is exactly constant (i.e., independent of time).

The most important factor influencing the solubility of a gas is pressure (4). The quantitative connection between solubility and pressure is generally expressed as Henry's law, which can be stated as the cc. of gas that will dissolve ideally in

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one cc. of film material is proportional to the pressure of the gas with which it is in equilibrium. If c is the cc. of the film material at equilibrium pressure p then

$$c = Sp \qquad \text{(Eq. 3)}$$

where S is a proportionality constant generally called the solubility coefficient or Henry's law constant and commonly defined in these connections as the cc. of gas that will dissolve ideally in one cc. of the film material when the pressure of the gas is one mm. of mercury.

If h is the thickness of the film the change in concentration gradient can be written as

$$\frac{dc}{dx} = \frac{c}{h} \qquad \text{(Eq. 4)}$$

and from Eq. 3 the concentration gradient becomes

$$\frac{dc}{dx} = \frac{c}{h} = \frac{Sp}{h} \qquad \text{(Eq. 5)}$$

combining Eqs. 1 and 5 and rearranging

$$\frac{Q \times h}{p} = -DS \qquad \text{(Eq. 6)}$$

putting $P =$

$$\frac{Q \times h}{p}$$

one has

$$P = -DS \qquad \text{(Eq. 7)}$$

P may be termed the permeability constant and may be defined as the cc. of gas at normal temperature and pressure that will pass per second through a unit area of the film one mm. thick when there is 1 cm. of mercury pressure between its faces.

Consequently the permeability of a film can be broken down into two contributing factors (3): the diffusion constant, which is a measure of the probability that each gas molecule will move in the direction of the concentration gradient, and the solubility coefficient, which measures the number of gas molecules per unit volume which contributes to this concentration gradient. The solubility coefficient according to Barrer (1) does not include the water molecules that are sorbed but only those that are ideally dissolved, and consequently this may or may not approximate the actual equilibrium solubility.

Anomalies observed in some of the present work, especially in the investigation of permeation through some of the waxes, are probably due to strong deviations from the assumption of a linear concentration gradient in the barrier phase, arising either from deviations from Henry's law of ideal solutions or from strong concentration dependency on the part of D. In an ideal steady-state concentration distribution across a membrane the drop in the concentration should be linear as shown in line A of Fig. 1, if the integral form of Fick's law is applicable. The concentration gradient in some of the waxes is, however, probably sigmoidal in character as shown in line B of Fig. 1, the gradient drop being small for the initial thickness and which then falls rapidly.

Henry's law, which can be shown to be a form of

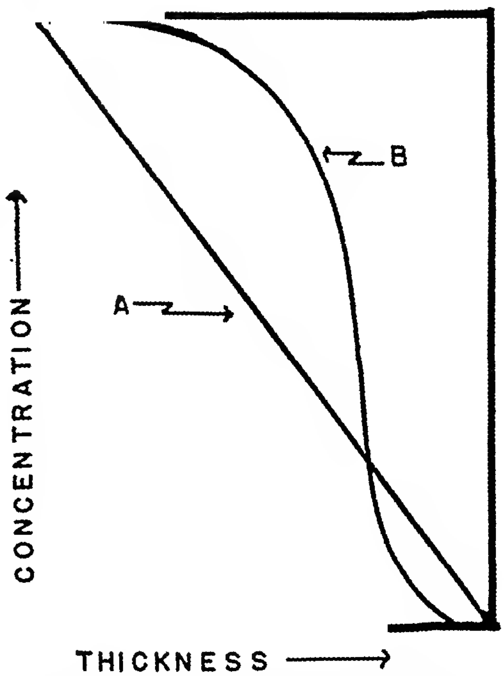

Fig. 1.—Hypothetical steady state concentration distribution across a membrane. A—linear. B—sigmoidal.

the general distribution law or of Raoult's law, is based on the ideal behavior of a gas, and any departure from ideality, such as those occurring with an easily compressible gas such as water vapor at low pressure or low temperature, will make the application of Henry's law invalid (5). Some causes of the departure from ideal behavior of a gas such as water vapor can be ascribed to self-association of the water molecules to form dimers or trimers in the barrier phase and diffusion as such, compound formation of the water with the material, and ionization of the vapor.

A suitable method of evaluating permeability, diffusivity, and solubility coefficients was developed by Barrer (6) based on a mathematical analysis by Daynes (7). A membrane of the polymer or other material is mounted in a diffusion cell and the permeation velocity is determined in the steady state as well as the rate at which that state is approached. The pressure on the ingoing side of the membrane is constant and is always much greater than that on the outgoing side.

The pressure on the outgoing side is plotted as a change in pressure *vs.* time curve. In most cases these curves emerge from the abscissa with a very small slope which continue over a period of time; the curve then gradually bends upwards and continues as a long straight line. There is an interval before the steady state can be approached due to finite diffusion velocity of the vapor within the membrane. Provided Fick's law is valid for the transport process, the intercept L made by the asymptotic curve on the axis of time is

$$L = \frac{h^2 1}{D(C_1 - C_2)} \frac{C_1}{6} + \frac{C_2}{3} - \frac{C_0}{2} \qquad \text{(Eq. 8)}$$

for a membrane of thickness h, where C_1 and C_2

are the concentrations of the solute within the ingoing side and outgoing side of the membrane, respectively, and C_0 is the initial uniform concentration of solute in the membrane Provided C_0 is zero and C_2 is much less than C_1 and also the diffusion coefficient is independent of concentration of gas in the film, then

$$L = \frac{h^2}{6D} \qquad \text{(Eq 9)}$$

L is the lag time, and h is the thickness of the membrane in cm Knowing the lag time, the diffusion coefficient D can be calculated using Eq 9 The permeability constant P can be obtained from the slope p(mm)/t(sec) of the straight line using the equation

$$P = \frac{p}{t} \times \frac{V}{760} \times \frac{h}{pxa} \qquad \text{(Eq 10)}$$

where V is the volume into which the gas expands after passing through the film, p is the vapor pressure of water in cm of mercury on the high pressure side of the film, h is the thickness of the film in cm, and a is the area of the film in sq cm Using the relation $P = DS$ the solubility coefficient S can be determined

EXPERIMENTAL

Apparatus

The design of the all glass apparatus used in the present study followed essentially that of Doty, *et al* (8) The setup consisted of two similar halves as shown in Fig 2, the left half in the diagram forming the high pressure or the water reservoir side and the right half the low pressure or the detection side The two parts were connected in use by means of a standard taper joint (50/50) The barrier film was mounted in the male part of the joint effectively separating the two sides, this is shown in detail in Fig 3

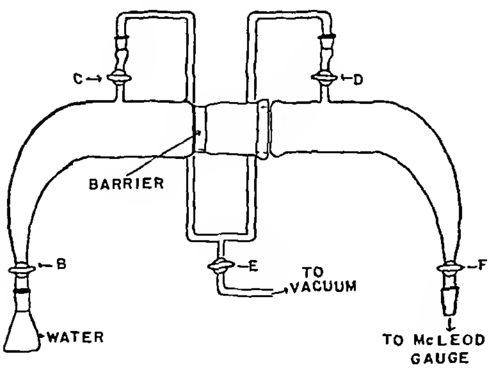

Fig 2 —A diagram of the diffusion cell

A McLeod gauge was used to measure the change in pressure on the low pressure side With a condensable gas such as water vapor serious errors can result from condensation of the vapor on the bulb and capillary of the gauge on raising the mercury to take a reading To avoid these errors the capillary of the gauge was jacketed with a water bath after the procedure of Doty, *et al* (8)

The problem of temperature control during

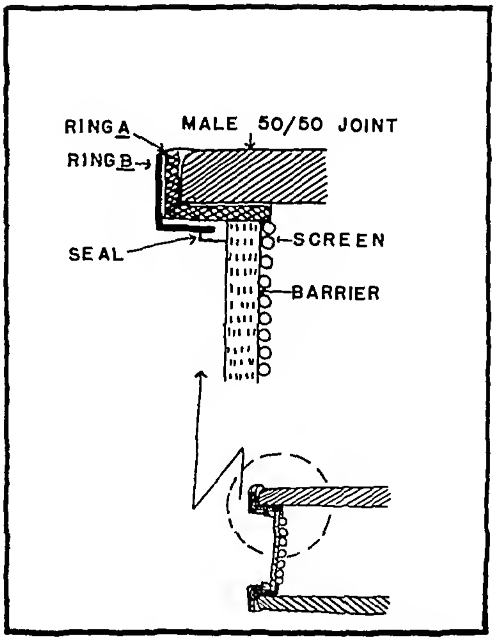

Fig 3 —Diagram of the barrier showing the method of sealing

measurement was largely solved by placing the entire apparatus in a constant temperature room The room was held to within ±0 3° of the set temperature by use of cooling and heating units controlled by a bimetallic sensing device

Procedure

Preparation of the films.—*Films from Cellulose Esters*—Cellulose ester films were cast from their solutions These were prepared by dissolving the polymer flakes by stirring and shaking overnight in solvents suggested by Antonides and DeKay (9), to make a 10 to 20% solution by weight

Plastic Films—These films were obtained directly from the manufacturers and were used as such

Glyceryl Stearate Films—Films of chromatographically purified (10) glyceryl stearates were made by casting on mercury

Measurement of Film Thickness.—The thickness of the plastic and cellulose ester films was measured with a micrometer caliper Twenty five readings of the thickness spread out over the surface of the film were taken and the average of these was used as the effective thickness

To prevent defacing the wax films, a slightly different procedure was followed in obtaining their thickness To insure uniformity of the cast, film thicknesses of regions around the center of the film were determined with the micrometer caliper A disk was then cut off from the center of the film and the thickness of the rim of the disk was measured with an optical micrometer

Procedure for Measurement of Permeability.—To make a determination, stopcocks B, C, D, and F (Fig 2) were fully open initially and stopcock E was used to control the initial rate of evacuation to prevent rupture of the film After deaerating the water in the flask by alternately freezing and allowing to come to room temperature, stopcock B was

shut off. The system was then evacuated to about 1×10^{-3} mm. of mercury pressure and the evacuation was carried on for about twenty-four hours to remove traces of water from the film. After this period stopcocks C, D, and E were shut off.

Before actual permeation measurements were begun, readings were taken for approximately an hour to make sure that no vapor was being given off from the films which would give erroneous results, and that the apparatus was not subject to leaks.

At zero time stopcock B was opened allowing the water vapor from the reservoir to come into contact with one face of the film. The change in pressure on the other side of the barrier was followed with the McLeod gauge.

RESULTS AND DISCUSSION

Permeation Through Various Materials

Permeation Through Cellulose Esters.—Results obtained in studying rates of permeation through films formed from cellulose acetate hydrogen phthalate, cellulose triacetate, cellulose acetate propionate, cellulose acetate butyrate, and cellulose acetate stearate are shown in Figs. 4, 5, and 6. The permeability and diffusion coefficients calculated from these plots as described above are given in Table I. The thickness of the films and some such values reported in the literature are also given for comparative purposes.

From the data it is evident that as the molecular weight of the ester groups in the cellulose or, equivalently, the proportion of the aliphatic content of the film increases, the permeability and the apparent diffusion coefficient calculated from lag time decrease markedly. The decrease in the former coefficient with increasing aliphatic content of the film can be probably ascribed to the decrease in the polarity of the film. The dependence of the rate of permeation on the polarity of the film arises from the solubility coefficient S of the permeability equation. In a more polar film the affinity of the water molecules for the barrier phase is greater, hence the solubility coefficient will be increased. This will increase the rate of permeation to the same extent. As the polarity of the film is decreased by the addition of large aliphatic substituents, the rate of permeation will, conversely, decrease.

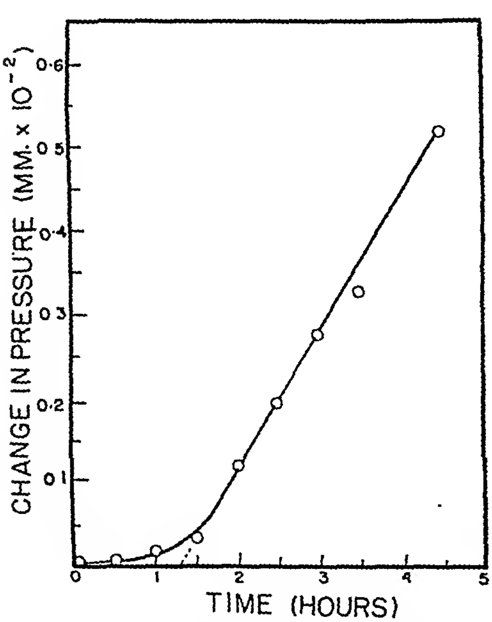

Fig. 4.—Plot showing permeation through cellulose acetate hydrogen phthalate (0.1322 cm. thick).

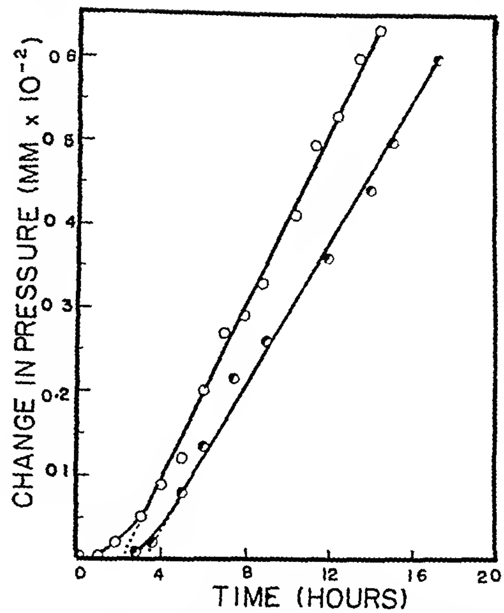

Fig. 5.—Plot showing permeation through ○— cellulose triacetate (0.1521 cm. thick) and ◐— cellulose acetate propionate (0.1625 cm thick).

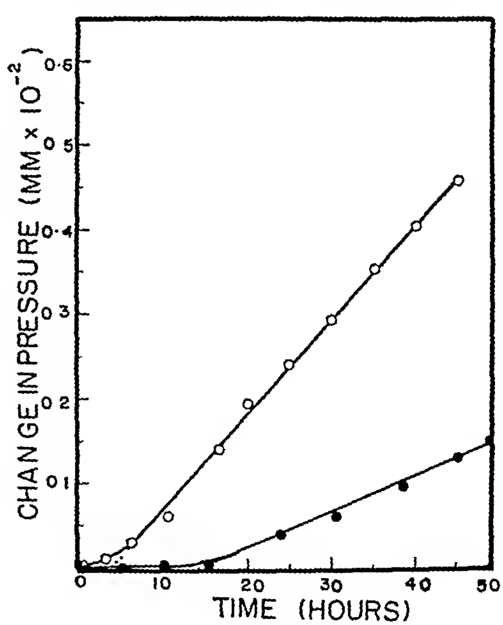

Fig. 6.—Plot showing permeation through ○— cellulose acetate butyrate (0.1666 cm. thick) and ●—cellulose acetate stearate (0.1631 cm thick).

Much has beeu written in the literature as regards the actual mechanism of passage of water through cellulose and cellulose-like materials. Waack (12), for instance, has ruled out the possibility that the water permeation through these materials is a capillary movement, since their void volume is extremely small. Doty, *et al.* (8), suggests the existence of very narrow crevices and slits between the crystallites of cellulose esters. It is thought that the water permeates through these slits and bonds with the polar groups available on the walls of the crystallites

However, as is evident from the data obtained in this study, the rate of permeation through cellulose esters is primarily governed by the availability of polar groups. The greater the number of these groups other factors notwithstanding, the greater the rate of passage of water.

Permeation Through Plastics.—Data obtained from a study of permeation of water vapor through Saran A, Koroseal, and polytrifluorochloroethylene are presented in Table II and Figs. 7 and 8. Again, for comparative purposes, data from the literature are included in Table II.

Examination of data obtained reveals that polytrifluorochloroethylene has a permeability coefficient one-tenth that of Saran A and approximately one-hundredth that of Koroseal. The diffusion coefficient of polytrifluorochloroethylene is also smaller than that of Saran A and Koroseal. This behavior of polytrifluorochloroethylene is of some interest. In all these plastics the basic structure is a hydrocarbon skeleton and there are no hydrophilic substituents or side chains, yet polytrifluorochloroethylene has a much lower permeability than the other two plastics.

The lower permeability of polytrifluorochloroethylene as compared to Saran A and Koroseal, in the absence of polar groups in all three plastics,

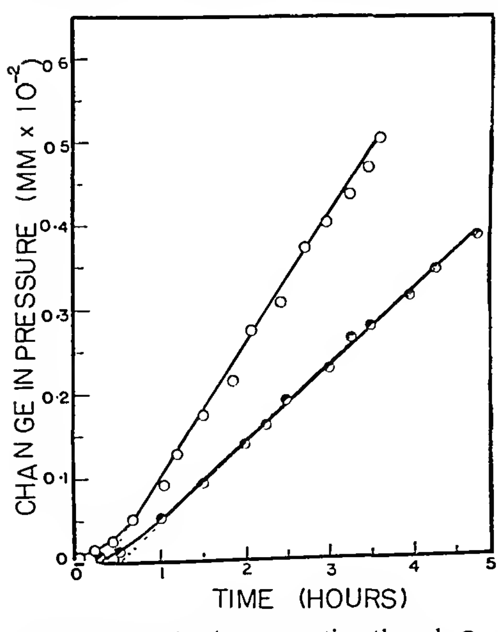

Fig. 7.—Plot showing permeation through O—Koroseal (0.0031 cm. thick) and ◐—Saran A (0.0048 cm. thick).

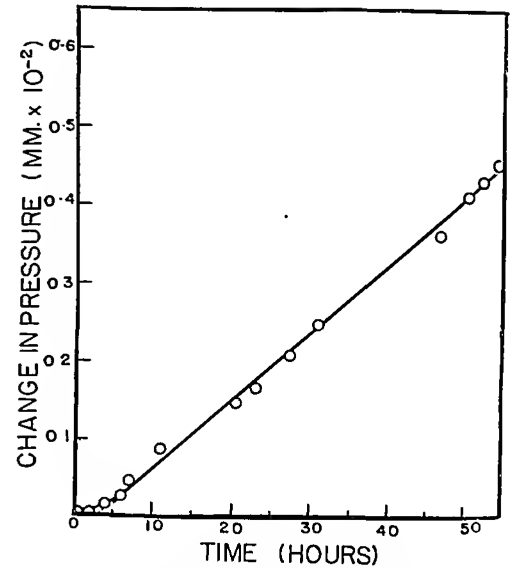

Fig. 8.—Plot showing permeation through polytrifluorochloroethylene.

can be attributed to more even packing of trifluorochloroethylene molecules or their extremely nonpolar character. In a polymer like polyvinylidene chloride, chlorine atoms are opposed by hydrogen atoms which differ greatly in size, making the packing of the molecules uneven. Frey, and others (13), have described polytr'fluorochloroethylene as polyvinylidene chloride in which all the hydrogen atoms are replaced by the larger fluorine molecules thus making the outward contour of the molecule more uniform. The uniformity of the molecules leads to a better packing of the material preventing any cracks, etc., through which the water may flow, consequently the rate of permeation through this material is extremely slow.

Permeation Through Glyceryl Waxes.—The permeability coefficient for glyceryl monostearate was determined as 12.9×10^{-8} and the diffusion coefficient was 194×10^{-8}. Attempts to determine the permeability and diffusion coefficients of pure glyceryl distearate and tristearate failed, as these films developed longitudinal cracks during their evaluation.

Comparison of the permeability coefficients of glyceryl monosterarate with those of cellulose esters studied again emphasizes the role of polar groups in permeation. Cellulose esters being more polar in general than glyceryl monostearate have larger permeability coefficients. For example, cellulose triacetate has a permeability coefficient approximately ten times that of glyceryl monostearate.

Dependence on Relative Humidity

Table III and Fig. 9 present data obtained on a study of variation of vapor pressure and relative humidity on the permeability and apparent diffusion coefficients of glyceryl monostearate.

For systems which follow Henry's law and also show independence of diffusion coefficient with respect to concentrations, experimentally deter-

TABLE I.—PERMEABILITY AND DIFFUSION COEFFICIENTS OF SOME CELLULOSE ESTERS

Material	Thickness, cm	Observed $D^a \times 10^5$	Observed $P^b \times 10^5$	Literature Values $D^a \times 10^5$	Literature Values $P^b \times 10^6$
Cellulose acetate hydrogen phthalate	0 1322	62 0	325		
Cellulose triacetate	0 1521	53 6	133	58 2	
Cellulose acetate propionate	0 1625	36 7	108		102 0 (11)
Cellulose acetate butyrate	0 1666	25 7	42		52 0 (11)
Cellulose acetate stearate	0 1631	8 5	10 5		

[a] Diffusion coefficient in cm.^{2} sec.$^{-1}$ [b] Permeability coefficient in cc./sec./cm.^{2}/cm. Hg/cm. thick.

TABLE II.—PERMEABILITY AND DIFFUSION OF COEFFICIENTS OF PLASTICS

Material	Thickness, cm	Observed Values $D^a \times 10^5$	Observed Values $P^b \times 10^5$	Literature Values $D^a \times 10^5$	Literature Values $P^b \times 10^6$
Saran A, polyvinylidene chloride	0 0048	0 21	0 05	0 125 (8)	0 07 (8)
Koroseal, polyvinyl chloride	0 0031	0 12	7 7		5 6 (8)
Polytrifluorochloroethylene	0 0048	0 02	0 006		

[a] Diffusion coefficient in cm.^{2} sec.$^{-1}$ [b] Permeability coefficient in cc./sec./cm.^{2}/cm. Hg/cm. thick.

TABLE III.—EFFECT OF CHANGE OF RELATIVE HUMIDITY ON GLYCERYL MONOSTEARATE

Humidity[a], %	$P^b \times 10^5$	$D^c \times 10^5$	$S^d \times 10^2$	Thickness, cm
100	12 9	194	6 64	0 1026
80	7 4	206	3 59	0 1021
52	5 2	165	3 15	0 1036
31	4 4	145	3 03	0 1073

[a] At 24 5° [b] Permeability coefficient in cc./sec./cm.^{2}/cm. Hg/cm. thick. [c] Diffusion coefficient in cm.^{2} sec.$^{-1}$ [d] Solubility coefficient in cc. of gas/cc. of film material.

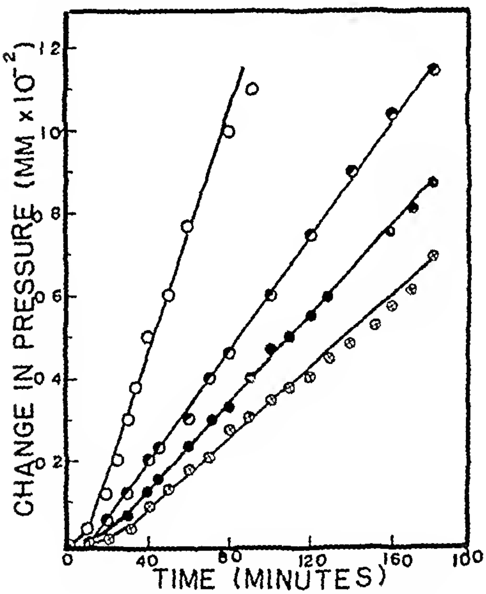

Fig 9.—Plot showing effect of change of relative humidity on permeation through glyceryl monostearate. ○—100% humidity (0 1026 cm. thick), ◐—80% humidity (0 1021 cm. thick), ●—52% humidity (0 1036 cm. thick), ⊗—31% humidity (0 1073 cm. thick).

mined values of P and D should be independent of the partial pressure of the penetrating vapor. This relationship apparently does not hold for glyceryl monostearate-water systems as is evident from the data. The rate of transfer of water vapor through this material appears to decrease much faster than the pressure of water vapor on this high pressure side.

This behavior unfortunately casts some doubt as to the absolute validity of the use of the lag time method in determining diffusion coefficients in these systems. It can be shown readily that if the permeability coefficients exhibit concentration dependency, then the diffusion coefficients determined in this fashion are only apparent. One must be concerned, for example, with the obvious tendency for materials of this type to bind water molecules which are not kinetically equally available for the diffusion process. This behavior alone will always give much too low a diffusion coefficient when calculated from lag time. Mathematical relationships in a similar system have already been developed but will be reported elsewhere.

The variation of the diffusion constants with change in vapor pressure in glyceryl monostearate appears to parallel the findings of Lowry and Kohman (14) in their work of solution of water in rubber. They state, "The solubility of water in rubber at vapor pressure below approximately 16 mm. at 25° is directly proportional to the first power of the pressure. At these pressures, then, the solubility obeys Henry's law, but at pressures higher than 16 mm., the solubility is much greater than the law would predict."

If a similar situation exists in the glyceryl monostearate system, Fick's linear diffusion law would be obeyed in the regions of low relative humidity. At high relative humidities the high concentration of water on the wet side of the membrane would result in lower apparent diffusion coefficient than would be expected.

Plots of permeability and diffusion coefficients *vs.* per cent humidity shown in Fig. 10 serve to emphasize the arguments presented above. As is evident from the figure, the apparent diffusion coefficient apparently reaches a maximum at 80% humidity and then decreases, instead of increasing as expected from theoretical considerations. The permeability coefficient on the other hand shows a greater rate of increase on passing from 80% hu-

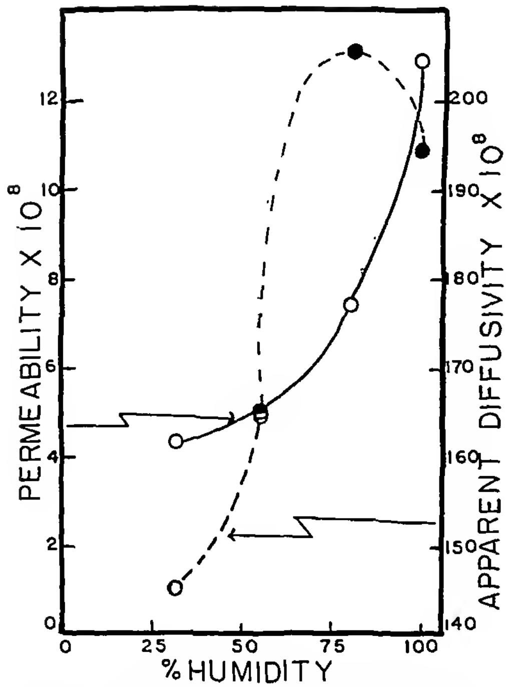

Fig. 10.—Plot showing variation of permeability and apparent diffusivity of glyceryl monostearate with change in relative humidity.

midity to 100%. The apparent reduction at 100% relative humidity in the apparent diffusion coefficient is probably due to a large increase in the number of water molecules bound by the wax phase.

Although a similar study on the cellulose esters was not carried out, a like situation probably exists. Both systems tend to sorb and bind water molecules significantly. In such cases diffusion coefficients estimated from lag time can only be considered apparent. It is presumed that if higher humidity was permitted to exist on the low pressure side of the barriers, the observed effective permeability would have been considerably higher.

Temperature Dependence of Permeation

Barrer (1) has conclusively shown that the permeation of gases and vapors through a film requires an energy of activation to make the molecules enter the pores and move along the path to the other surface. This he has expressed in an Arrhenius type of equation

$$P = P_0 e^{-\frac{E_p}{RT}} \qquad \text{(Eq. 11)}$$

where P is the permeability constant. P_0 is a factor, independent of temperature, which is proportional to the number of molecules that are available to enter the pore structure and also to the probability that a molecule having a sufficient energy will actually enter this pore. P_0 may also be defined as the permeability constant at absolute zero. E_p is the energy of activation for permeation. R is gas constant and T absolute temperature.

The equation for temperature dependence of diffusion is also given by an Arrhenius type of equation and can be written as

$$D = D_0 e^{-\frac{E_D}{RT}} \qquad \text{(Eq. 12)}$$

where D is the diffusion coefficient, D_0 is a constant independent of temperature or the diffusion coefficient at absolute zero, and E_D is the energy of activation for diffusion.

If P behaves as Eq. 11 and D behaves as Eq. 12, and since $P = -DS$, it is necessary that the solubility coefficient S exhibit the same functional variation with temperature. Hence it would require that

$$S = S_0 e^{\frac{H}{RT}} \qquad \text{(Eq. 13)}$$

where S_0 is a constant and H is the heat of solution of water vapor in the membrane.

The variation of the diffusion and permeability coefficients of glyceryl monostearate with temperature is shown in Table IV and Fig. 11.

The activation energy for permeation was

TABLE IV.—EFFECT OF TEMPERATURE VARIATION ON THE PERMEABILITY AND APPARENT DIFFUSIVITY THROUGH GLYCERYL MONOSTEARATE

Temperature, °C.	$D^a \times 10^8$	$P^b \times 10^8$	Thickness, cm.
16.9	91.5	8.9	0.1047
24.3	194	12.9	0.1006
29.5	333	16.2	0.1039

[a] Diffusion coefficient in cm.² sec.⁻¹.
[b] Permeability coefficient in cc./sec./cm.²/cm. Hg/cm. thick.

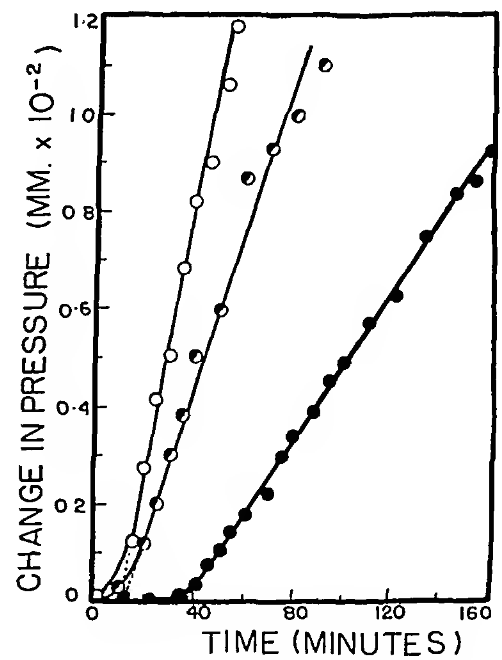

Fig. 11.—Plot showing temperature dependence of permeation through glyceryl monostearate. ○—G. monostearate (0.1047 cm. thick) at 16.9°. ◐—G. monostearate (0.1006 cm. thick) at 24.3°. ●—G. monostearate (0.1039 cm. thick) at 29.5°.

obtained from the slope of a plot of log of permeation *vs.* the reciprocal of the absolute temperature shown in Fig. 12.

The activation energy of permeation was found to be 7,700 cal./mole and the apparent activation energy for diffusion was 16,400 cal./mole. By difference, the heat of solution was calculated and the value obtained was 8,700 calories, a very reasonable value.

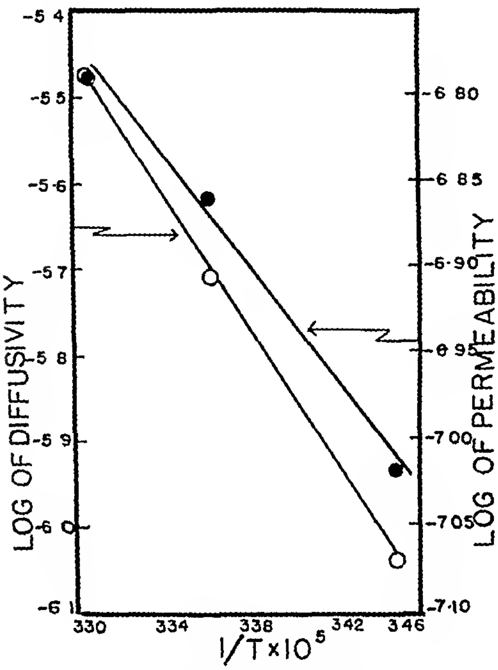

Fig. 12.—Plot of log of diffusivity and log of permeability *vs.* the reciprocal of absolute temperature from which the activation energies were calculated.

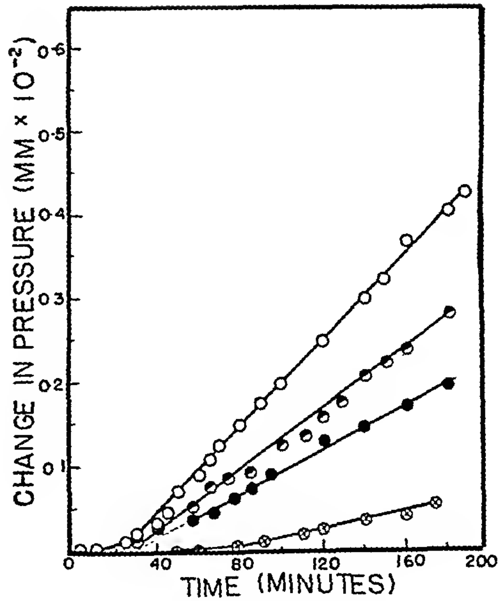

Fig. 13.—Plot showing permeation through glyceryl monostearate admixed with different concentrations of white wax. O—G. monostearate + 10% white wax (0.2172 cm.). ◐—G. monostearate + 20% white wax (0.2021 cm.). ●—G. monostearate + 30% white wax (0.2094 cm.). ⊗—G. monostearate + 40% white wax (0.2136 cm.).

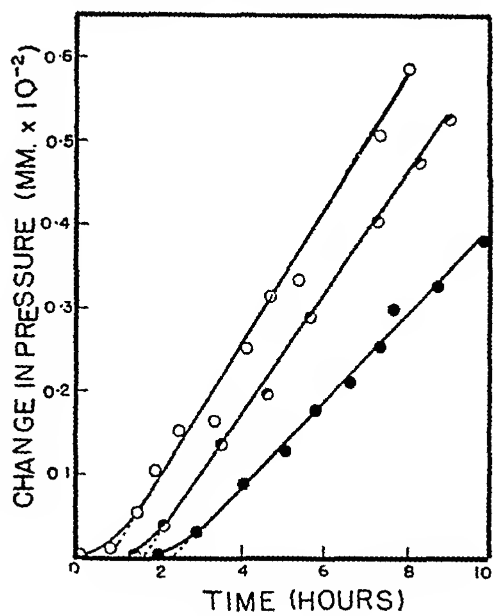

Fig. 14.—Plot showing permeation through glyceryl distearin admixed with various concentrations of white wax. O—G. distearate + 10% white wax (0.2031 cm.). ◐—G. distearate + 20% white wax (0.2081 cm.). ●—G. distearate + 30% white wax (0.2060 cm.).

Permeation Through Mixtures of Glyceryl Stearate and White Wax

Addition of a relatively impermeable substance like white wax to the glyceryl stearates tends to enhance their water resistance. This is evident from Tables V, VI, and VII, and also from Figs. 13, 14, and 15, where experimental results obtained on the several stearates admixed with white wax are presented.

Decrease of the permeability coefficient of glyceryl stearates on progressive increase of concentration of white wax can again be ascribed to the decrease in the effective solubility coefficient of the permeability equation. The solubility coefficient would decrease since the addition of white wax would eliminate some of the active centers where the water molecules could bond, i. e., the free hydroxyl groups in the glycerides.

Since glyceryl monostearate has a higher number of these active centers the rate of the decrease of the permeability coefficient would be much greater than glyceryl di- or tristearate. This is borne out from a comparison of data in Tables V, VI, and VII.

It is apparent from Table VI that the apparent diffusion coefficient of glyceryl monostearate remains essentially constant with increase of concentration of white wax up to 30%, after which it drops markedly. This may be due to possible formation of a two-phase system by the two components corresponding to that concentration of white

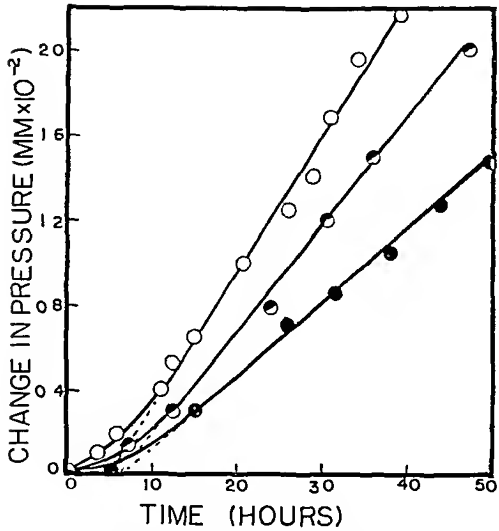

Fig. 15.—Plot showing permeation through glyceryl tristearate admixed with various concentrations of white wax ○—G. tristearate + 20% white wax (0 1089 cm) ◐—G tristearate + 30% white wax (0 1125 cm) ●—G tristearate + 50% white wax (0 0993 cm)

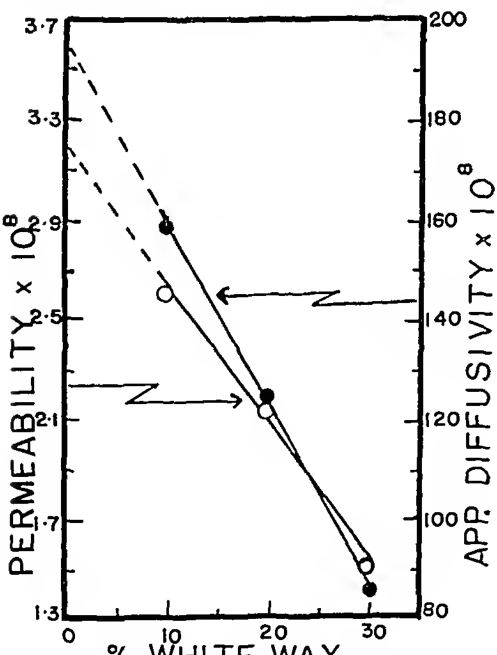

Fig. 16.—Plots showing extrapolation to zero per cent white wax concentration from which approximate values of the permeability and apparent diffusion coefficients of glyceryl distearate were obtained.

wax necessary to form a more or less continuous phase.

Of some interest is the behavior of the diffusion coefficient of glyceryl di- and tristearate on addition of increasing concentration of white wax. Tables VI and VII indicate that the diffusion coefficient

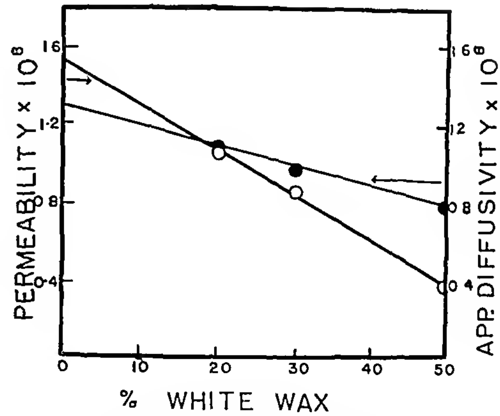

Fig. 17.—Plots showing extrapolation to zero per cent white wax concentration from which approximate values of the permeability and apparent diffusion coefficients of glyceryl tristearate were obtained.

falls with the addition of white wax although the rate of fall with tristearin is much greater than with distearin.

Approximate values of the permeability and apparent diffusion coefficients of glyceryl di- and tristearate were obtained by extrapolating plots, shown in Figs. 16 and 17, of per cent of white wax *vs.* these constants to zero white wax concentration. As mentioned earlier, direct determination of these constants was not possible due to the brittleness of di- and tristearin. The values obtained are given in Table VIII.

TABLE V.—EFFECT OF ADDITION OF WHITE WAX TO GLYCERYL MONOSTEARATE

White Wax, %	Thickness, cm	$D^a \times 10^8$	$P^b \times 10^8$
10	0 2172	403	5 5
20	0 2021	404	3 5
30	0 2094	405	2 3
40	0 2136	171	1.0

[a] Diffusion coefficient in cm ^{2} sec $^{-1}$
[b] Permeability coefficient in cc /sec /cm ^{2}/cm Hg/cm thick

TABLE VI.—EFFECT OF ADDITION OF WHITE WAX TO GLYCERYL DISTEARATE

White Wax, %	Thickness, cm.	$D^a \times 10^8$	$P^b \times 10^8$
10	0 2031	159	2 6
20	0 2081	125	2.1
30	0 2060	86.4	1 5

[a] Diffusion coefficient in cm ^{2} sec $^{-1}$
[b] Permeability coefficient in cc /sec /cm ^{2}/cm Hg/cm thick

TABLE VII.—EFFECT OF ADDITION OF WHITE WAX TO GLYCERYL TRISTEARIN

White Wax, %	Thickness, cm	$D^a \times 10^8$	$P^b \times 10^8$
20	0 1089	1	1
30	0 1125	0 97	0.85
50	0 0993	0 78	0.37

[a] Diffusion coefficient in cm ^{2} sec $^{-1}$
[b] Permeability coefficient in cc /sec /cm ^{2}/cm. Hg/cm. thick

TABLE VIII.—PERMEABILITY AND DIFFUSION COEFFICIENTS OF GLYCERYL DI- AND TRISTEARATE

	$D^a \times 10^9$	$P^b \times 10^9$
G distearate	204	3.21
G tristearate	13	1.54

[a] Diffusion coefficient in cm.[2] sec.[-1]
[b] Permeability coefficient in cc./sec./cm.[2]/cm. Hg/cm. thick.

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Some Physical and Crystallographic Properties of the Ouabain Hydrates*

By JAGDISH TRIVEDI, JOHN W. SHELL†, and JOHN A. BILES

Different hydrates of ouabain have been isolated. The physical and crystallographic properties of the hydrates as well as the X-ray powder diagrams are reported and discussed. The solubility of ouabain is reported for two different water-alkanol systems.

THE EXAMINATION of the available data on the isolation of and the stability of possible hydrates of ouabain indicates the data to be confusing. There is also confusion existing with respect to the optical crystallographic data of the hydrates. Thus, the purpose of this communication is to clarify the methods for isolating the different hydrates, to report the optical crystallographic properties of the hydrates, and to record the X-ray powder diffraction data.

The crystallographic properties of ouabain have been reported by Keenan and co-workers (1, 2), Shell and Witt (3), and Kofler (4). Keenan and co-workers reported the isolation of three different hydrates of ouabain. Ouabain with 19.2 per cent water was recrystallized from hot water; and ouabain containing 11.9 per cent water was recrystallized from 95 per cent ethanol. The refractive indexes of these hydrates were recorded (1). Shell and Witt determined the optical crystallographic data for ouabain recrystallized from ethanol.

Kofler reported the isolation of the decahydrate and octahydrate of ouabain when recrystallized from a cooled aqueous solution. He stated that the trihydrate was isolated from hot water or when acetone was added to the octahydrate and allowed to stand for several hours. Siewart (5) reported the isolation of the trihydrate of ouabain by dissolving 0.5 Gm. of ouabain in 17 cc. of dioxan and 0.3 cc. of water and allowing to stand at room temperature for about two days. Kofler further reported the isolation of the dihydrate from acetone, alcohol, and aqueous sodium chloride (1 per cent) solution. The addition of sodium chloride to an alcoholic solution was a simple means of isolating the dihydrate. Kofler stated that the ouabain dihydrate was unstable in an aqueous solution but stable in the dry state.

It was concluded from a study of the literature that the octahydrate, the trihydrate, and the hydrate from alcohol were stable, whereas the decahydrate was unstable and the dihydrate was stable under certain given conditions. The melting points of all the hydrates excepting the trihydrate were reported to be 180–185°. The trihydrate melted at 240–245°.

The solubility of ouabain in water was reported by Arnaud (6) and Richaud (7). Additional solubilities are listed in the U. S. P. XV (8) and the Merck Index (9). Several workers have reported ouabain to be stable (10–13). Berry reported that ouabain was stable over a wide pH range (14).

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Abstracted in part from a dissertation by Jagdish Trivedi submitted to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.
† Research Associate, The Upjohn Co.

EXPERIMENTAL

Materials —Ouabain, U S P , ethanol, U S P , and isopropanol, N F , were used in preparing the alkanol water solutions for the solubility determinations Ethanol, U S P , methanol, C P , and dioxane, C P , were used as crystallizing solvents Sodium chloride, C P , was used in crystallization of ouabain Karl Fischer reagent was prepared according to U S P XV directions

Apparatus —A Karl Fischer apparatus similar to that described in U S P XV was used to determine the water content of some ouabain hydrates The water content of the different phases was also determined using an Abderhalden apparatus, charged with phosphorus pentoxide The boiling solvent was bromobenzene, b p 150–155° The solubility of the ouabain in the different alkanol solvents was determined using a rotating wheel apparatus which held 20 cc vials The wheel was rotated by a motor and was submerged in a constant temperature bath Rotation was continuous for eight hours

Crystallization of the Ouabain Hydrates.—The ouabain was placed in the solvent for crystallization An excess of ouabain was used, and the solvent was heated above the crystallization temperature The solution was allowed to cool to the temperature desired for crystallization following filtration After a sufficient amount of the ouabain had crystallized in the container, the heterogenous system was closed to prevent further evaporation of the solvent The system was allowed to equilibrate for twenty four hours In this manner, ouabain was recrystallized at 0° and at intervals of 10° from 20 through 90° The recrystallization at elevated temperatures was done in an oven

Ouabain was recrystallized from ethanol by saturation of a heated solution and allowed to cool Ouabain was allowed to crystallize from 6% water in dioxane by heating the solution, filtering, and allowing to cool Ouabain was crystallized from 97% methanol and also from a 50% ethanol solution containing 1% sodium chloride

Determination of Water Content in the Ouabain Hydrates —In the initial determination of per cent water in the hydrates, the Karl Fischer method of water determination was used However, the method required the use of rather large amounts of the ouabain Therefore, the loss of weight of heating the hydrates in the Abderhalden apparatus was checked against the Karl Fischer method The precision was close enough to justify the use of determination of water content by loss in weight The recovery of ouabain was greater using the loss in weight method

Optical Crystallographic Properties.—The optical crystallographic data were determined by means of a petrographic microscope A Scopelite (Clay Adam Co , N Y) containing a 25 watt bulb and blue filter was used as a light source The method of determination of the data was similar to that described by Biles, Witt, and Poe (15)

Photomicrographs —The crystals were mounted on the petrographic microscope A Bausch and Lomb photomicrographic camera model L was used in obtaining the photomicrographs

Melting Points.—The melting points were determined using a Kofler melting point block mounted on a microscope stage The crystals were observed under 80 × magnification

X-ray Powder Diagrams —The diameter of the camera was such that the mm distance between arcs on the film was equivalent to a degree Copper radiation, filtered by nickel, was used A sliding scale with an attached vernier was used to measure the distance between a given pair of arcs

Solubility Determinations and Assay.—Duplicate samples were used in determining the solubility of ouabain in ethanol water and isopropanol-water solutions The percentages w/w of the solutions were determined by specific gravity determinations using a Westphal balance, and refractive index determinations using an Abbe refractometer The values were compared with the values reported in the International Critical Tables (16)

An excess of ouabain was placed in a 20 cc vial The solvent was added and the vial stoppered with a rubber seal Eight vials were placed in a plastic wheel The wheel was rotated in a constant temperature bath for eight hours The vials were removed and the solution filtered The filtrate was rapidly collected and the solution diluted and assayed using the U S P XV procedure for assay A Beckman model B spectrophotometer was used for the colorimetric method of analysis

RESULTS AND DISCUSSION

Ouabain Hydrates —Six different hydrates of ouabain were isolated The melting point, water content, and the temperature of isolation are recorded in Table I The temperature water composition phase diagram for the hydrates isolated from water is illustrated in Fig 1

The nonahydrate was isolated by crystallization at temperatures of 0–15° The nonahydrate was not observed when ouabain was crystallized from

TABLE I —WATER CONTENT, M P , AND TEMPERATURE OF ISOLATION OF THE OUABAIN HYDRATES

Hydrate	Water Calculated %	Water Experimental [a] %	M p °C	Temperature of Isolation °C
Ouabain $9H_2O$	21 71	21 93	190–195	0–15
Ouabain $8H_2O$	19 78	20 13	190–195	15–28
Ouabain $4^1/_2H_2O$	12 18	12 10	185–187	20–30[c]
		11 84[b]		
Ouabain $4H_2O$	10 98	10 88	190–195	20–30[d]
Ouabain $3H_2O$	8 46	8 43	240–247	20–30[e]
Ouabain $2H_2O$	5 80	6 02	185–195	28–90
Ouabain			190–195	150[f]

[a] Determined by loss of weight of water in Abderhalden apparatus at 150° Average of duplicate determinations [b] Karl Fischer method of determination [c] From 95% ethanol [d] From 97% methanol [e] From dioxane containing 6% water [f] Obtained by heating ouabain recrystallized from ethanol in Abderhalden apparatus at 150°

water at room temperature. This phase evidently corresponded to the decahydrate reported by Kofler (4). Attempts were not successful in isolating the decahydrate. The octahydrate was isolated at room temperature. The octahydrate and the dihydrate existed in equilibrium at 28° in aqueous solution. This was repeated several times since Schwartze (1) reported isolating the trihydrate from hot water and Kofler (4) stated that the trihydrate was the most stable hydrate in saturated aqueous solution at a temperature of about 70°. When the dihydrate was isolated from the aqueous solution, dried between filter paper, and the material subjected to drying in the Abderhalden, there was a water loss of 8.12% However, the X-ray powder patterns for the material dried between filter paper and the material subjected to drying at 130° were the same. Therefore, it might be possible that Schwartze and Kofler considered the material isolated at 70° to be the *trihydrate*, whereas the present investigation indicates that the dihydrate contains excess water which is not part of the repeat lattice. The dihydrate was isolated from aqueous solutions at temperatures of 28° up to 90°. Above 90° isotropic resin-like material was isolated.

Kofler's work was verified with respect to isolation of ouabain from ethanol. Similar results were reported by Schwartze. Water composition studies indicated that the crystal contained an equivalent of 4.5 moles of water per mole of ouabain. When ouabain was crystallized from 97% methanol, water loss indicated that the crystal contained an equivalent of 4 moles of water per mole of ouabain. The optical crystallographic properties were very similar in all respects except for the principal refractive indexes. The X-ray patterns for these powders from ethanol and methanol were the same. It was concluded, therefore, that the one-half mole of water in the crystals from ethanol was not part of the repeat lattice. This extra water may be held by entrapment by hydrogen bonding. Therefore, it may be possible that optical crystallographic properties can detect solvated phases to which X-ray powder patterns are insensitive.

Optical Crystallographic Data.—The data are recorded in Table II. The properties of the hydrate from ethanol corresponded to those values recorded by Keenan. The properties of the hydrate from 97% methanol were similar to those recorded by Shell and Witt. The octahydrate was determined to be monoclinic with the α- and β-refractive indexes corresponding to the values reported by Keenan. However, Keenan reported the octahydrate to be tetragonal; the octahydrate appears to be pseudotetragonal. Kofler reported the refractive indexes of the trihydrate. His values indicated the crystal to be optically negative. On orientation of the bisectrix, the trihydrate was determined to be positive. The only point of difference was the reported value for the alpha index. The anhydrous form was determined to be isotropic. The refractive index corresponded to that reported by Keenan—namely 1.565. When the dihydrate was subjected to drying in the Abderhalden at 150°, the optical crystallographic data were redetermined. Some data indicated the crystals differed from the dihydrate before drying. X-ray powder patterns of the two crystals were the same.

The crystal habits and systems are very similar for most of the hydrates. These data justify the statement made by Kofler that on heating, the optical properties do not change. Futhermore, this explains the failure to notice changes in crystalline phases on the Kofler block and the similarity

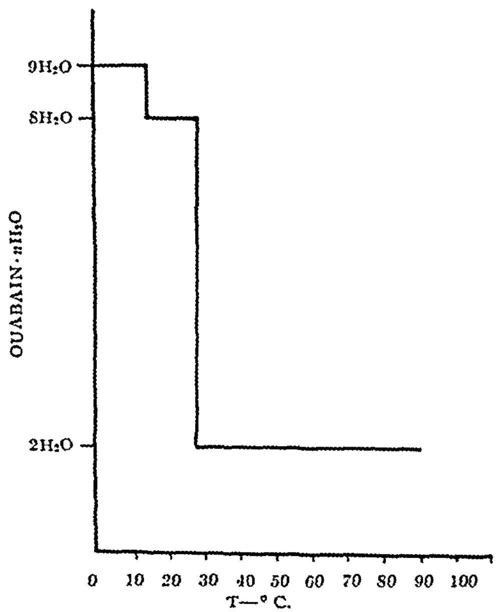


Fig. 1.—Temperature-water composition phase diagram for ouabain hydrates.

TABLE II.—THE OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF THE HYDRATES OF OUABAIN

Hydrate	Crystal Habit	Crystal System	Optic Orientation[c]	Optic Sign	Axial Angle[a]	Dispersion	Common Orientation	Refractive Indexes α	β	γ
Ouabain·$9H_2O$	Tabular	Orthorhombic	XX‖a YY‖b	−	59°	None	Bxa, off Centered	1.502	1.521	1.532
Ouabain·$8H_2O$	Tabular	Monoclinic	XX‖b YY∧c 33°	−	38°	Crossed (Bxo)	Bxa, off Centered	1.521	1.525	1.528
Ouabain·$4^1/_2H_2O$	Columnar	Orthorhombic	XX‖a, YY‖b	+	55°	None	Bxo	1.531	1.542	1.581
Ouabain·$4H_2O$	Tabular	Orthorhombic	XX‖a, YY‖b	+	33°	None	Bxo	1.533	1.536	1.570
Ouabain·$3H_2O$	Acicular	Orthorbombic	XX‖a, YY‖b	+	74°	v > r, axial	Bxo, off Centered	1.571	1.582	1.602
Ouabain·$2H_2O$	Columnar	Orthorhombic	XX‖a, YY‖b	+	41°	v > r, axial	Bxa, off Centered	1.557	1.560	1.582
Ouabain·$2H_2O$[b]	Columnar	Orthorhombic	XX‖a, YY‖b	+	43°	v > r, axial	Bxo	1.550	1.553	1.573

[a] 2V, calculated. [b] The dihydrate dried at 150° for four hours [c] Assigned according to crystal habit.

in melting points of the different phases. The formation of the anhydrous phase from the trihydrate and dihydrate is least practical using the Abderhalden apparatus and a boiling solvent of 150°.

X-ray Powder Patterns.—The interplanar spacings in Å. were calculated and recorded in Table III. The three strongest lines observed on the films are ranked in order of intensity by 1, 2, and 3. Examination of ouabain $4^1/_2H_2O$ (from ethanol) and ouabain $\cdot 4H_2O$ (from methanol) powder *d*-distances indicate similar lattices The same is true for the dihydrate and the dihydrate dried in the Abderhalden It has been suggested that the X-ray powder patterns are insensitive to some solvated phases This suggests water entrapment by hydrogen bonding It is thought that proton resonance spectra will clarify the differences between the optical crystallographic data of the hydrates showing the same repeat lattices A future communication concerning this problem is planned

Solubility of Ouabain in Alkanol-Water Systems.—The solubility of ouabain in ethanol-water solutions and isopropanol-water solutions at 25° was determined and shown in Fig 2 At 25° ouabain

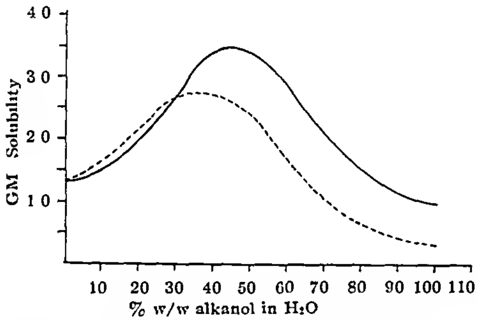


Fig 2—Solubility of anhydrous ouabain in ethanol-water (———) and isopropanol-water (- - - -) solutions at 25°.

was found to be 1.34% soluble in water. The solubility recorded in The Merck Index is 1 Gm. in about 75 ml. of water. The solubility of ouabain in 95% ethanol is greater than 1% which is reported in the Merck Index. The maximum solubility of ouabain is 3 51% in 42% w/w ethanol-water and 2 7% in 38.42% w/w isopropanol-water solutions

Recently Moore (17) discussed the relationship of maximum solubility in blended solvents and the dielectric constant. His work was applied to this study. At 25° the dielectric constants for ethanol, isopropanol, and water are, respectively, 24 3, 18 3, and 78 54. Assuming an ideal solution, the dielectric constant for a 42% w/w ethanol in water is 55.76 and the dielectric constant for a 38 42% w/w isopropanol in water is 55 38. This indicates that maximum solubility of ouabain in a blended solvent occurs when the dielectric constant for that solvent is 55 38–55.76.

SUMMARY

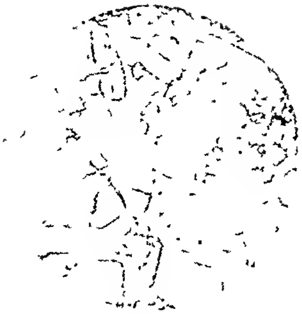
Six different hydrates of ouabain have been isolated. The temperature-water composition phase diagram is reported to describe the phases.

The optical crystallographic properties of the ouabain hydrates have been determined and compared with values reported in the literature for some of the hydrates. The anhydrous ouabain is isotropic.

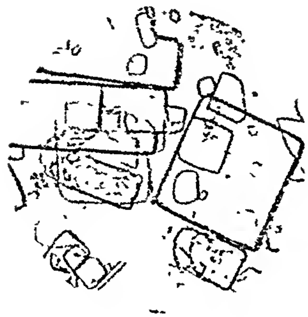
The *d*-distances for the different hydrates were determined using powders and using copper radiation filtered with nickel. The X-ray powder patterns for ouabain $\cdot 4^1/_2H_2O$ (from ethanol) and ouabain $\cdot 4H_2O$ (from methanol) are the same. The X-ray powder patterns for ouabain $\cdot 2H_2O$ and the dihydrate which lost 8.12 per cent water on drying at 150° for four hours are the same. It is possible that the optical crystallographic

TABLE III—THE *d*-DISTANCES FOR THE OUABAIN HYDRATES USING CU K-ALPHA RADIATION

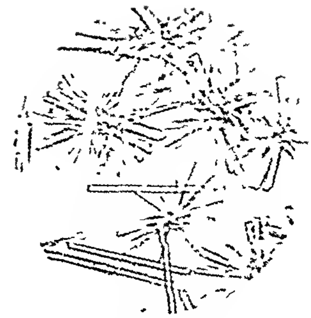
Ouabain $2H_2O$	Ouabain $2H_2O$ Dried 4 Hr at 130°, Abderhalden	Ouabain $3H_2O$	Ouabain $4H_2O$	Ouabain $4^1/_2H_2O$	Ouabain $8H_2O$
13 80	13 70	11 53	18 39	14 02	13 69
10 21	10 27	9 60	14 24	11 11	11 04
8 93	9 06	8 42	11 04	9 21	10 21
7 86	7 93	7 33	9 16	7 52	9 25
6 68 (3)	6 75 (3)	6 70	8 26	6 63 (1)	8 30 (1)
6 17 (2)	6 19 (2)	6 34 (1)	7 49	6 17	7 62
5 75	5 80	5 79	6 68 (1)	5 90 (2)	6 99
5 18	5 17	5 40 (2)	6 15	5 71	6 73 (2)
4 87 (1)	4 88 (1)	4 90	5 90 (2)	5 09 (3)	6 17
4 38	4 58	4 42 (3)	5 50	4 87	5 59
4 16	4 37	3 89	5 01 (3)	4 70	5 16 (3)
3 71	4 16	3 63	4 77	4 50	4 82
3 40	4 00	3 45	4 50	4 27	4 55
3 09	3 85	3 17	4 32	4 05	4 27
2 63	3 73	4 01	4 08	3 73	4 02
2.38	3.40	2 88	3 93	3 52	3 83
	3 27	2 79	3 70		3 56
	3.08	2 70	3 54		3 42
	2 89	2 54	3 42		
	2 71	2 36	3 26		
	2 61	2 26	2 07		
	2 53	2 21	2 92		



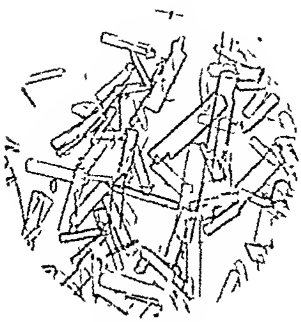
OUABAIN $9H_2O$



OUABAIN $8H_2O$

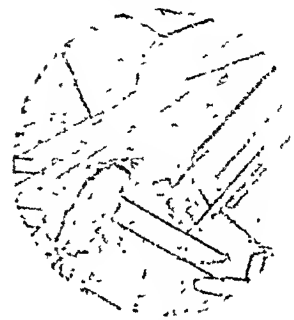


OUABAIN $4\frac{1}{2}H_2O$

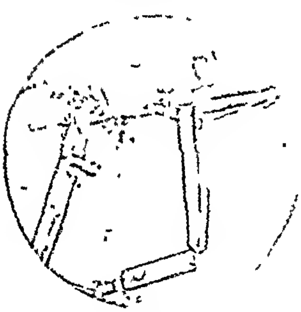


OUABAIN $4H_2O$

OUABAIN $3H_2O$



OUABAIN $2H_2O$



OUABAIN $2H_2O$

Fig 3 —Photomicrographs showing crystal habits of ouabain hydrates, dried at 150° for four hours All magnifications ×40

properties distinguish different solvated phases of ouabain to which the X-ray powder patterns are insensitive The entrapped water is evidently not a part of the repeat lattice but held by hydrogen bonding. The entrapped water is held tightly since the hydrates are stable at room temperature

Photomicrographs of the ouabain hydrates are shown in Fig 3 The close similarities of the phases are easily demonstrated with the photomicrographs

The solubility of the ouabain in ethanol-water and isopropanol-water solutions have been reported It was concluded that ouabain is most soluble in blended solvents having a dielectric constant of 55.38–55.76 at 25°.

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Formation of Complexes By N,N′-Dimethyl-diketobenzodiazines in Nonaqueous Solutions*

By AMIN F. HADDAD†, BARTLEY J. SCIARRONE‡, and TAKERU HIGUCHI

A study has been made of the complexing behavior in carbon tetrachloride of three N,N′-dimethyl-diketobenzodiazines with phenols. 1,3-Dimethylbenzoyleneurea in carbon tetrachloride forms insoluble complexes with hydroquinone and resorcinol, and a soluble complex with phenol. Catechol, on the other hand, forms, at low concentrations, a soluble complex; and then an insoluble complex starts to form after the solubility limit of catechol is reached. 2,3-Diketo-1,4-dimethylquinoxaline in carbon tetrachloride has a tendency to form soluble complexes with resorcinol and favors formation of various complexes with catechol and phenol of the soluble and insoluble types. No complexing tendency was observed in the case of hydroquinone. Within the concentration limits of the phenols used, no complex formation was observed in the systems containing 2,3-dimethylphthalylhydrazide and catechol, resorcinol, or hydroquinone; phenol, however, formed a soluble complex.

In recent years a large number of studies concerned with complex formation in aqueous systems have been reported. Many of these studies were designed in an attempt to elucidate the nature of the bonding forces responsible for complex formation and to determine the chemical and physical properties of these so-called molecular complexes.

The nature of these forces, especially in aqueous solutions, is still somewhat in doubt. However, it is generally believed that the main forces responsible for complex formation are basically electrostatic in nature and that the particular type of the interaction force or forces involved may be of various kinds depending on the component entering into the formation of the individual complex molecule

In explaining complex formation in aqueous solutions, Higuchi and Lach (1) pointed out that on the basis of electrostatic attraction between the interacting groups alone the extent of complex formation would be extremely small since the competing water dipoles are present in such high concentration. They believe that the tendency for complex formation in such a system is rendered favorable by the interaction of the hydrophobic, lipophilic portions of the component molecules in a manner similar to micelle formation. These hydrophobic units are thought to be "squeezed out" of the water phase by the high internal pressure of water leading to a high degree of interaction. This is explained by the same authors, on the basis that the squeezing together of the hydrocarbon portions of the reactants permits the water molecules originally surrounding these units to form additional hydrogen bonds; the net effect is an apparent high degree of interaction between the relatively inert hydrocarbon groups.

Earlier works (1–4) indicated that caffeine is particularly capable of forming molecular complexes with pharmaceutical acids, esters, salts, and certain aromatic and nonaromatic nitrogenous compounds. The complexing behavior of caffeine has been attributed (4) to the carbonyl group activated by the adjacent nitrogen atom. Consequently in an attempt to provide a better understanding of the intermolecular forces involved in the formation of molecular complexes and the structural requirements favoring complex formation in aqueous systems, a series of compounds having structural similarities to caffeine have been chosen and their complexing tendencies are being studied (9) in aqueous solutions using phenolic substances as the complexing agent. Results of these studies will be reported at a later date. The present study is concerned with the complexing behavior of three of these compounds in a nonaqueous media in order to eliminate the possibility of the occurrence of the competitive reactions, phenol-substrate and water-substrate interactions which are believed to exist in aqueous systems due to the high polarity of water. Accordingly carbon tetrachloride has been chosen as the nonpolar solvent in the present preliminary study. It is hoped that through the use of nonpolar solvents, the solute-solvent interactions might be eliminated, thus permitting a better understanding of the intramolecular and intermolecular forces existing between the components of a molecular complex formed both in aqueous and nonaqueous solutions.

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EXPERIMENTAL

Reagents.—Phenol, analytical reagent grade. Resorcinol, U. S. P. recrystallized. Hydroquinone, photo-purified, Mallinckrodt. Catechol, resublimed, m. p. 104.5–106.5°. 1,3-Dimethylbenzoyleneurea: prepared in the laboratory by the methylation of benzoyleneurea with dimethylsulfate in an alkaline medium, m. p. 164–166°. Benzoyleneurea was prepared by the process of Bogert and Scatchard (5), slightly modified. 2,3-Diketo-1,4-dimethylquinoxaline: 2,3-diketoquinoxaline was first prepared (6) and then methylated by using dimethylsulfate in an alkaline solution (7), m. p. 254–256°. 2,3-Dimethylphthalylhydrazide was prepared (8) by the condensation of phthalic anhydride and symmetrical dimethylhydrazine, m. p. 175–177°. Carbon tetrachloride, analytical reagent grade.

Procedure.—The solubility method was employed in determining the extent and nature of possible complex formation in the systems under investigation The substrate and the complexing agents, except phenol, were accurately weighed in the dry state into 10-ml. Kimble clear-glass ampuls. Ten milliliters of carbon tetrachloride were then added. In the case of phenol, accurately measured volumes of a standard solution were added to the substrate and the final volume was made up to 10 ml. by the addition of the required volume of carbon tetrachloride. The hermetically sealed ampuls were immersed in a constant temperature water bath equipped with a rotating bar to which the racks, holding ten ampuls each, were fastened. The ampuls were allowed to equilibrate at 30° for periods ranging from forty to forty-six hours.

After equilibration the ampuls were opened and the required volume of solution was withdrawn by means of a volumetric pipet fitted with a piece of latex-rubber tubing enclosing a plug of glass wool as filter. In the case of 2,3-diketo-1,4-dimethylquinoxaline, a layer of glass powder between the two glass wool layers was necessary to filter off the colloidal precipitate. The resulting clear solution was suitably diluted with carbon tetrachloride to the required volume in volumetric flasks and the final dilution analyzed on Model 11 ms Cary recording spectrophotometer. In order to eliminate the interference of the phenols, which start absorbing at about 310 mμ wavelength, the absorbance values of the substrate were read at 324 mμ wavelength.

RESULTS AND DISCUSSIONS

1,3-Dimethylbenzoyleneurea.—The complexing tendencies of this compound with catechol, resorcinol, hydroquinone, and phenol were studied in carbon tetrachloride. The results are reported here below.

Catechol.—The interaction between 1,3-dimethylbenzoyleneurea and catechol is shown in Fig. 1. In order to build up the phase diagram five runs were carried, each on a different day. The phase diagram shows that at low catechol concentration a straight line relationship is obtained between the dimethylbenzoyleneurea and catechol. The increase in the solubility of the dimethylbenzoyleneurea is due to the formation of a soluble complex which continues until the solubility limit (about 1.379×10^{-2} moles per liter) of catechol is reached.

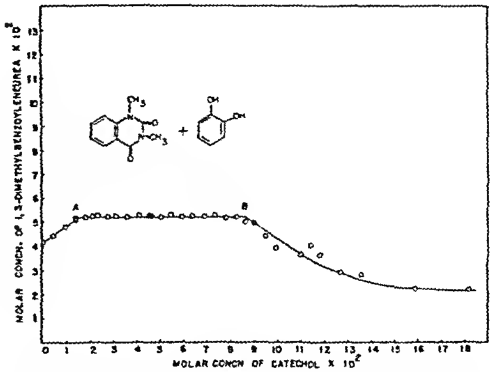

Fig. 1.—Phase solubility plot showing interaction between 1,3-dimethylbenzoyleneurea and catechol in carbon tetrachloride.

Point A in Fig. 1 represents the saturation point of the solution with regard to the complex. Further addition of catechol results in formation of more complex which precipitates from the already saturated system. The precipitation continues until the excess of the dimethylbenzoyleneurea added to the system has been utilized and the solubility curve begins to descend at point B. This drop is due to the complete consummation of all excess dimethylbenzoyleneurea and subsequent depletion of this material in solution.

The stoichiometric ratio of the components of the complex which was formed in the plateau region can be calculated from the phase diagram. This is possible because the excess of free dimethylbenzoyleneurea found at point A is equivalent to the amount being converted to the complex during the interval between points A and B in Fig. 1. The corresponding amount of the catechol being converted to the complex is equal to that entering into the complex during the same interval.

Calculations:

Dimethylbenzoyleneurea content of complex formed in the plateau region = total dimethylbenzoyleneurea added to system—dimethylbenzoyleneurea in solution at point A = $7.97 \times 10^{-2} - 5.22 \times 10^{-2} = 2.75 \times 10^{-2}$

Catechol content of complex in the same region = $8.62 \times 10^{-2} - 1.45 \times 10^{-2}$ (read from diagram) = 7.17×10^{-2}

Then the stoichiometric ratio

$$= \frac{\text{Dimethylbenzoyleneurea}}{\text{catechol}} = \frac{2.75 \times 10^{-2}}{7.17 \times 10^{-2}} = \frac{1}{2.6} \text{ or } \frac{2}{5.12}$$

The above results indicate that, most probably, the complex formed is a 2:5 complex. According to the phase diagram other complexes are possible.

Resorcinol.—The phase diagram in Fig. 2 indicates that there is a definite interaction between 1,3-dimethylbenzoyleneurea and resorcinol. There is a very slight initial rise in the curve, which could be attributed to experimental error, indicating that the complex formed is extremely insoluble in carbon tetrachloride, since no significant initial increase in the apparent solubility of dimethylbenzoyleneurea is observed, in contrast to the dimethylbenzoyleneurea-

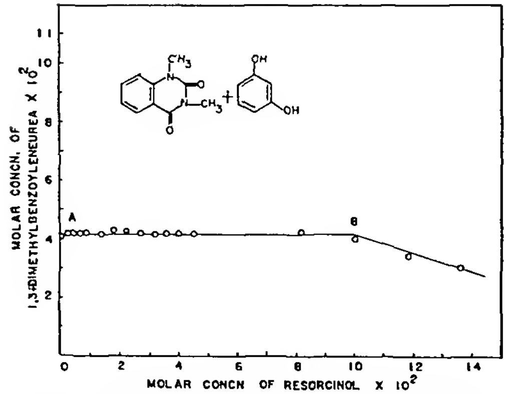

Fig 2.—Phase solubility plot of 1,3-dimethylbenzoyleneurea and resorcinol in carbon tetrachloride.

catechol system discussed above It is worth mentioning here that resorcinol and hydroquinone are extremely insoluble in carbon tetrachloride as compared to catechol, which is soluble to the extent of about 1 379 × 10^{-2} moles/L. Phenol is very soluble in carbon tetrachloride This difference in the solubility behavior of these phenols in carbon tetrachloride might explain the difference in the solubility behavior of the corresponding complexes formed.

The ratio of the components of the complex formed in the plateau region can be calculated from the plot in Fig 2 This is possible because the plateau region represents the amount of resorcinol entering into complex formation during this interval and the corresponding amount of dimethylbenzoyleneurea being converted to the complex The amount of the latter component is equal to that present in the form of free undissolved solid in the system at zero resorcinol concentration The system between points A and B is invariant, which means that as the dimethylbenzoyleneurea is removed in the form of insoluble complex it is replaced from the excess present in the system. When all the excess of dimethylbenzoyleneurea is used up the dimethylbenzoyleneurea titer drops as shown in the diagram at point B. Therefore, by determining the quantities of resorcinol and dimethylbenzoyleneurea used in the plateau region, the stoichiometry of the complex formed during the same interval can be calculated

Calculations:

Dimethylbenzoyleneurea content of complex formed in the plateau region = total dimethylbenzoyleneurea added to system—dimethylbenzoyleneurea in solution at point A = 6 75 × 10^{-2} − 4.17 × 10^{-2} = 2.58 × 10^{-2}

Resorcinol content of complex during same interval = 10.035 × 10^{-2}

$$\text{Then the stoichiometric ratio} = \frac{\text{dimethylbenzoyleneurea}}{\text{resorcinol}}$$

$$= \frac{2.58 \times 10^{-2}}{10.035 \times 10^{-2}}, \text{ or roughly } \frac{1}{4}$$

Hydroquinone.—This compound is extremely insoluble in carbon tetrachloride. The phase diagram in Fig. 3 shows a weak binding activity between hydroquinone and dimethylbenzoyleneurea. From the phase diagram it is possible to calculate for the ratio of the components of the complex formed in the plateau by employing the same procedure described above under the dimethylbenzoyleneurea-resorcinol system.

Calculation:

$$\frac{\text{Dimethylbenzoyleneurea content of the complex formed in the plateau region}}{\text{Hydroquinone content of complex in the same region}} = \frac{6.84 \times 10^{-2} - 4.05 \times 10^{-2}}{3.67 \times 10^{-2}}$$

$$= \frac{2.79 \times 10^{-2}}{3.67 \times 10^{-2}} = \frac{1}{1.32}$$

Phenol.—The influence of phenol on the solubility of 1,3-dimethylbenzoyleneurea is shown in Fig. 4. The diagram shows that dimethylbenzoyleneurea and phenol form a soluble complex in the presence of zero to 5 334 × 10^{-2} molar concentration used of the latter component. The molecular ratio of the complex cannot be calculated. Assuming a 1:1 stoichiometric ratio the value of the stability constant of this system is 2.44.

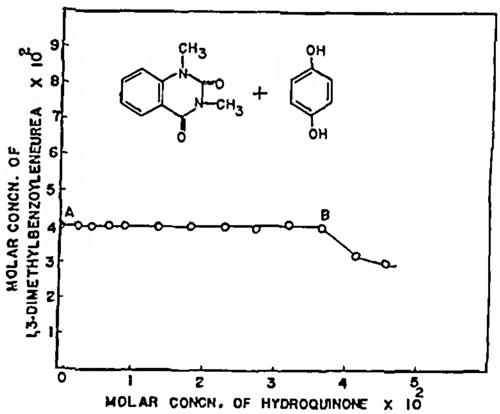

Fig 3.—Phase solubility plot of 1,3-dimethylbenzoyleneurea and hydroquinone in carbon tetrachloride.

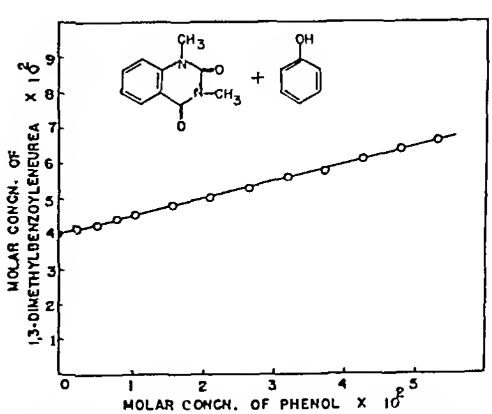

Fig 4.—Phase solubility plot of 1,3-dimethylbenzoyleneurea and phenol in carbon tetrachloride.

2,3-Diketo-1,4-dimethylquinoxaline.—A study was carried out on the possibility of complex formation in systems containing 2,3-diketo-1,4-dimethylquinoxaline and catechol, resorcinol, hydroquinone, and phenol The following is a brief discussion of the results

Catechol—It was observed that 2,3-diketo-1,4-dimethylquinoxaline has a tendency to form with catechol several forms of molecular complexes of the soluble and the insoluble types No diagram of this system is reproduced in the present communication

Resorcinol—This compound forms a soluble complex with 2,3-diketo-1,4-dimethylquinoxaline as is shown in Fig. 5 It is significant to note the high proportion of resorcinol consumed for every unit rise in the solubility of the quinoxaline compound.

Hydroquinone.—No complexing tendency was observed in the system containing hydroquinone and 2,3-diketo-1,4-dimethylquinoxaline

Phenol—The phase diagram shown in Fig. 6 indicates that systems containing phenol and 2,3-diketo-1,4-dimethylquinoxaline favor formation of various forms of complexes of the soluble and the insoluble types dependent upon the molar concentration of phenol present in the system.

2,3-Dimethylphthalylhydrazide.—Within the concentration limits of the phenols used no complex formation was observed in the systems containing

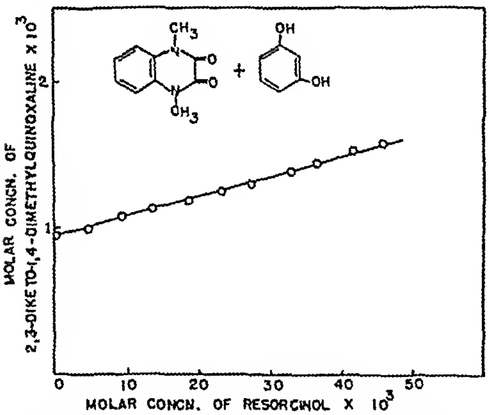

Fig 5—Phase solubility plot of diketodimethylquinoxaline and resorcinol in carbon tetrachloride

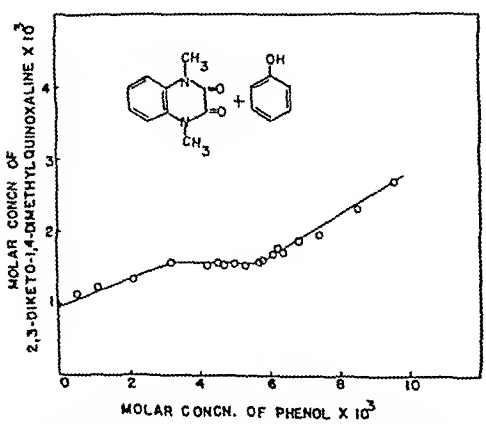

Fig. 6.—Phase solubility plot of diketodimethylquinoxaline and phenol in carbon tetrachloride.

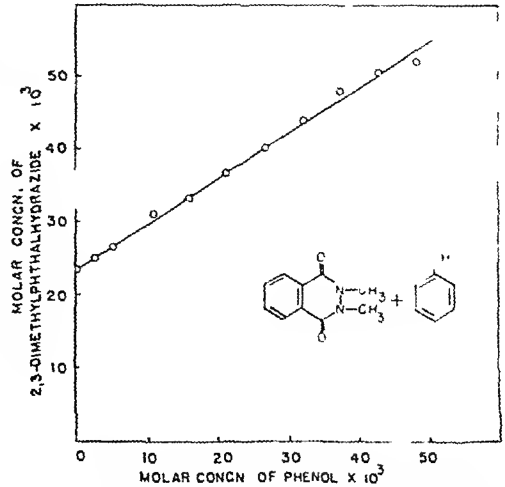

Fig 7—Phase solubility plot of dimethylphthalylhydrazide and phenol in carbon tetrachloride.

2,3-dimethylphthalylhydrazide and catechol, resorcinol, and hydroquinone Phenol, however, has a tendency to form soluble molecular complexes with the phthalylhydrazide compound as shown in Fig 7

CONCLUSION

The experimental findings presented above show that, of the three, N, N′-dimethyl-diketobenzodiazines studied, 1,3-dimethylbenzoyleneurea has the strongest tendency to form complexes in carbon tetrachloride with catechol, hydroquinone, resorcinol, and phenol 2,3-Diketo-1,4-dimethylquinoxaline forms complexes with catechol, resorcinol, and phenol but not with hydroquinone On the other hand, 2,3-dimethylphthalylhydrazide forms complexes with phenol, but no complexes were observed with catechol, hydroquinone, and resorcinol

Although the experimental findings of this preliminary study are insufficient to elucidate the nature of the structural relationship and binding forces existing between the components of the molecular complexes, they do indicate that complex formations in the systems studied are possible in nonaqueous solvents where the competitive forces manifested by water in aqueous systems are absent.

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Complexes Formed in Aqueous Solutions By Sarcosine Anhydride; Interactions With Organic Acids, Phenols, and Aromatic Alcohols*

By JOHN W. POOLE† and TAKERU HIGUCHI

It has been shown that the interactions between sarcosine anhydride, a cyclic dipeptide, and forty-six organic proton donor compounds result in the formation of both soluble and insoluble molecular complexes. The studies illustrate the importance of the aromatic and hydrophobic character of the molecule, as well as the nature of the polar groups and their positions, on complexing activity. The stoichiometry of a large number of insoluble complex species found were determined from analysis of phase diagrams and chemical analysis of the solid materials. Apparent stability constants were determined for the soluble complexes formed in the investigation. It is suggested that many biochemical and drug actions are mediated through formation of complexes of this type.

PREVIOUS INVESTIGATIONS (1–10) have demonstrated the formation of molecular complexes in aqueous solutions by the interaction of compounds capable of donating protons with such agents as caffeine, polyethylene glycols, polyvinylpyrrolidone, and a series of water-soluble amides. The present study is concerned with similar complex formation between sarcosine anhydride and various acidic substances which were chosen to illustrate the structural factors favoring these interactions. The complexing tendencies of various acids, phenols, and alcohols with sarcosine anhydride were observed for this purpose by means of solubility studies. In addition, where insoluble complexes were formed the stoichiometric ratios and melting points of these products were determined.

Ability of sarcosine anhydride to form various molecular association compounds in aqueous solution was thought to be of particular pharmaceutical interest for two reasons. It is, in a sense, a dipeptide which may be expected to present in aqueous phase a surface crudely comparable to that of a protein (enzyme). As has been previously speculated, interactions of this type may well mediate drug and biochemical reactions. Secondly, because of its amino acid origin, sarcosine anhydride may prove to be an acceptable nontoxic complexing agent useful in modifying solubility and stability properties of certain drugs from a formulation standpoint.

Specifically, the relative tendency of sarcosine anhydride to form molecular complexes with the following compounds were determined: *ortho-*, *meta-*, and *para-*hydroxy benzoic acids, benzoic acid, aspirin, *ortho-*phthalic acid, 4-aminosalicylic acid, 2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, gallic acid, 8-hydroxyquinoline, gallacetophenone, phloroglucinol, 2,5-dihydroxybenzoic acid ethanolamide, 2,3-dihydroxyquinoxaline, *ortho-*, *meta-*, and *para-*aminophenols, salicylamide, salicyl alcohol, salicylamine, 1,5-naphthalenediol, 2,7-naphthalenediol, hydroquinone, 2,6-dihydroxytoluene, hexylresorcinol, *ortho-*, *meta-*, and *para-*xylylene glycols, suberic acid, pimelic acid, adipic acid, rutin, oxytetracycline, melamine, chloramphenicol, quinine hydrochloride, picric acid, phenobarbital, and L-tyrosine.

The purpose of this study was (*a*) to observe the tendency of sarcosine anhydride to associate with proton-donor compounds with the possibility of interpreting these data in terms of the stereochemical factors favoring complex formation; (*b*) to note whether a compound like sarcosine anhydride, with little aromatic character, would be capable of entering into any strong specific interactions; and (*c*) to obtain general information useful in the elucidation of the whole field of complexing.

EXPERIMENTAL PROCEDURE

The experimental procedure employed in this study was similar to that described in an earlier publication by Higuchi and Lach (6). Small glass vials were used as the reaction vessels with 10, 5, or 1 ml. of solvent added, depending on the initial solubility and/or availability of the proton-donor substance. A spectrophotometric analysis was utilized in a majority of the studies. In the remaining cases, the interactions were followed by titration of the acidic compound. Distilled water was employed as the general solvent, however 0.001 *N* sulfuric acid was used with the carboxylic acids to suppress any ionization of the acids. A 20% sodium chloride solution was utilized with *meta-*xylylene glycol to substantially reduce the initial solubility of

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this compound When the donor substance was especially labile to oxidation, 0 1% sodium bisulfite was added to the system

The relationship between concentration and volume fraction of sarcosine anhydride was obtained from specific gravity measurements The specific gravities of several sarcosine anhydride solutions were determined by the pycnometer method

All of the compounds employed in this investigation were of reagent grade or recrystallized from suitable solvents

Synthesis of Sarcosine Anhydride.—Sarcosine anhydride, 1,4 dimethyl-1,5 diketopiperazine, was prepared essentially by the method described by Bilek, *et al* (11), for the preparation of N,N′-diethyl-2,5 diketopiperazine The ethyl ester of sarcosine was obtained by treating sarcosine hydrochloride with absolute ethanol and dry HCl gas The resulting ester hydrochloride was transformed to the free ester by the use of anhydrous ammonia and separated by vacuum distillation The ethyl ester of sarcosine was placed in a sealed tube and heated for thirty six hours at 180° The product was purified by recrystallization from absolute ethyl alcohol with overall yields of 30–40% The recrystallized compound has m p 145–146°, reported (12) m p 149°

RESULTS AND OBSERVATIONS

General Behavior.—In a majority of the cases a straight line is obtained when the apparent solubility of the acidic compound in water is plotted against the amount of sarcosine anhydride present The phase diagrams for the interactions of sarcosine anhydride with the aminobenzoic acids, shown in Fig 1, are typical for this type system The principal complex being formed, if we attribute the increase in solubility to complex formation, is then directly dependent on the sarcosine anhydride concentration in these ranges Because the concentration of the free acid substance remains constant, the stoichiometric ratio of the complexes formed in these studies cannot be determined from the phase diagrams The only requirement for the straight line plot is for the complex formed to be unimolecular with respect to the sarcosine anhydride It is evident that if the stoichiometry of these complexes is unknown the true stability constants cannot be calculated If some stoichiometry is assumed, however, the apparent stability constants can be evaluated by use of Eq 1

$$K_m = \frac{(A_nB)}{(A)^n(B)} \qquad \text{(Eq 1)}$$

where (A_nB) is the concentration of complex, (A) is the concentration of free acidic substance, (B) is the concentration of free sarcosine anhydride, and n is the number of molecules of acidic substance associated with each sarcosine anhydride molecule The details for this calculation have been shown by Higuchi and Zuck in a previous publication (3)

The stability constants based on the assumption of a 1 1 molecular complex were calculated for nearly all of the systems investigated to facilitate a comparison of the complexing tendency of sarcosine anhydride with similar complexing agents previously studied, as well as to give an indication of the order of complexing activity demonstrated by the substances studied These constants are included in Tables I, III, V, VI, and VII

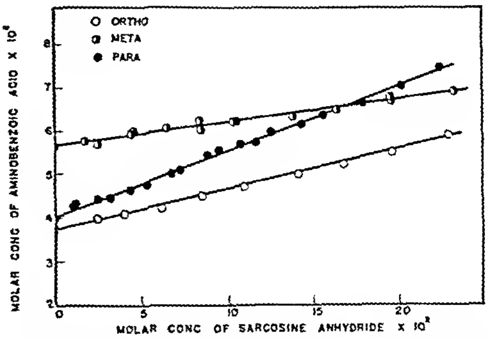

Fig 1—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of the aminobenzoic acids in water at 25°

Since the majority of insoluble complexes obtained in this investigation had a stoichiometric ratio of two acid molecules to one molecule of sarcosine anhydride, it is reasonable to expect a predominance of a 2 1 molecular ratio in the soluble complexes formed in solution The stability constants calculated on this basis appeared to give a more accurate description of the overall complexing ability of sarcosine anhydride The constants determined on this basis are also included in the tables previously mentioned Since there is a possibility of several simultaneous equilibria in solution, the nature of the interaction cannot be accurately described

Plateau regions in the phase diagram were found with substances which formed more insoluble complexes This invariant region is a result of the solution being saturated with respect to both the complex and the acidic compound The interaction between limited excess of 2,5 dihydroxybenzoic acid and sarcosine anhydride, for example, results in an insoluble complex, which is illustrated by the phase diagram in Fig 2 The stoichiometry of complexes of this type can be calculated from the phase diagrams according to the method of Higuchi and Lach (6) Thus, it can be seen from Fig 2 that the concentration of acid in solution remains constant along the line AB At point B all the excess solid acid has been precipitated as complex, beyond this point 2,5 dihydroxybenzoic acid is being depleted from solution The amount of sarcosine anhydride repre-

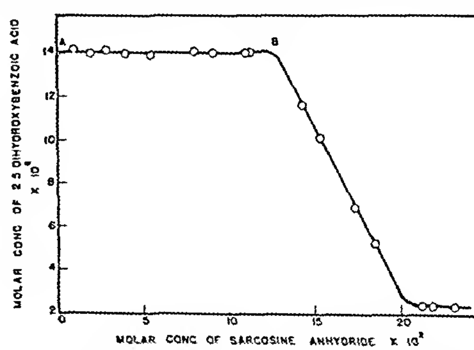

Fig 2—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 2,5 dihydroxybenzoic acid in water at 25° The system contained a total of 0 390 mole/L of 2,5 dihydroxybenzoic acid in and out of solution.

TABLE I —INTERACTION OF VARIOUS AROMATIC CARBOXYLIC ACIDS WITH SARCOSINE ANHYDRIDE AT 25°

Compound	Orig Solubility, Molar Concn × 10²	$K_2{}^a$, (liter) (mole)	$K_3{}^b$ (liter) (mole)	Increase in Solubility With a 1% Sarcosine Anhydride Soln, %
p-Hydroxybenzoic acid	4 31	5 4	54[c]	30
o-Hydroxybenzoic acid	1 75	4 3	150	30
m-Hydroxybenzoic acid	5 85	3 6	27[d]	21
p-Aminobenzoic acid	4 03	4 2	49	26
o-Aminobenzoic acid	3 73	2 6	34	17
m-Aminobenzoic acid	5 63	1 1	10	7
Isonicotinic acid	4 73	0	0	0
Benzoic acid	2 85	2 9	50	19
Aspirin	2 27	3 1	65	19
o-Phthalic acid	3 90	2 2	29	16
4-Aminosalicylic	1 00	5 1	250	36
2,4-Dihydroxybenzoic acid	3 75	3 4	63[e]	..
3,4-Dihydroxybenzoic acid	8 92	9 6	38[f]	..
2,5-Dihydroxybenzoic acid	13 6		..	..
3,5-Dihydroxybenzoic acid	66 0			..
Gallic acid	7 78	64	.	..

[a] Apparent stability constant calculated on a 1 1 basis
[b] Apparent stability constant calculated on a 2 1 basis, determined from one point
[c] Value from downcurve is 51
[d] Value from downcurve is 25
[e] Value from downcurve is 66
[f] Value from downcurve is 40

sented by the length of the plateau is equal to that entering into the insoluble complex during that interval The difference between the total 2,5-dihydroxybenzoic acid added to the system and that found in solution at point A is the corresponding amount of acid converted to the insoluble complex

Thus,

sarcosine anhydride content of complex formed in the plateau region $= 12\ 6 \times 10^{-2}$ moles/L (from diagram)

2,5-dihydroxybenzoic acid content = total acid-acid in solution at point A
$= 39\ 0 \times 10^{-2} - 14 \times 10^{-2}$
$= 25\ 0 \times 10^{-2}$ moles/L

Then the stoichiometric ratio equals 2,5-dihydroxybenzoic acid content $= \dfrac{25\ 0 \times 10^{-2}}{12\ 6 \times 10^{-2}} = 2\ 0$

The results indicate that two molecules of acid are associated with each molecule of sarcosine anhydride

In those instances where the descending portion of the phase diagram is a straight line extending closely to the base line, it is also possible to obtain the stoichiometric ratio of the complex in the following manner If the downcurve is extrapolated to zero concentration of the acidic substance, then the difference between the concentration of complexing agent at the start of the downcurve and that at the extrapolated point, minus the amount of complexing agent present as soluble complex, is equal to the amount of complexing agent needed to interact with the free proton-donor substance in solution Therefore, the stoichiometry of the complex can be obtained by dividing the concentration of the proton-donor compound in solution by the concentration of complexing agent needed to interact with the donor substance in solution It is possible to demonstrate this calculation for the interaction described by the phase diagram in Fig. 2

concentration of sarcosine anhydride at extrapolated point $= 20\ x \times 10^{-2}$ moles/L.

concentration of sarcosine anhydride at start of downcurve $= 12\ 6 \times 10^{-2}$ moles/L — sarcosine at extrapolated point anhydride at start of downcurve—sarcosine anhydride present as soluble complex
$= 20\ 2 \times 10^{-2} - 12\ 6 \times 10^{-2}$
$= 7\ 6 \times 10^{-2}$ moles/L

2,5-dihydroxybenzoic acid in solution $= 14\ 0 \times 10^{-2}$ moles/L.

Then the stoichiometric ratio equals 2,5-dihydroxybenzoic acid content/sarcosine anhydride content $= \dfrac{14.0 \times 10^{-2}}{7\ 6 \times 10^{-2}} = 1\ 8$

The results indicate that the reaction product is probably a two-to-one complex.

The stoichiometric ratios obtained by analysis of the phase diagrams were confirmed by isolation and chemical analysis of the insoluble reaction products Tables II and IV list the stoichiometric ratios determined by the various methods along with the melting points of the insoluble complexes

Where the plateau region of the phase diagram is preceded by a rise in the curve, as exhibited in the interactions of sarcosine anhydride with *meta-* and *para*-hydroxybenzoic acids shown in Fig. 4, it is possible to obtain the stability of the complex formed For interactions of this type the descending portion of the curve can also be used to determine the stability constant by use of Eq 1 in the following manner:

The complex in solution, (A_nB), is determined from the difference between the original solubility of the acidic substance and the maximum concentration it attains at the plateau; (B), the free complexing agent in solution = (total complexing agent

TABLE II.—STOICHIOMETRIC RATIOS AND MELTING POINTS OF THE INSOLUBLE COMPLEXES FORMED BETWEEN AROMATIC CARBOXYLIC ACIDS AND SARCOSINE ANHYDRIDE

Compound	Molecular Ratio Found of Acid: Sarcosine Anhydride — Plateau[a]	Extrapolation[b]	Chem. Anal.[c]	Probable Stoichiometric Ratio	M. p. of Complex, °C
m-Hydroxybenzoic acid	1.9:1		2.2:1	2:1	154–156
p-Hydroxybenzoic acid	2.4:1		2.4:1	2:1	188–190
2,4-Dihydroxybenzoic acid	2.1:1		1.8:1	2:1	187–188
2,5-Dihydroxybenzoic acid	2.0:1	1.8:1	1.8:1	2:1	183–184
3,4-Dihydroxybenzoic acid	2.2:1	2.2:1	1.9:1	2:1	188–192
3,5-Dihydroxybenzoic acid	5.9:1	5.6:1	6.3:1	6:1	212–220
Gallic acid	6.0:1	5.6:1	5.2:1	6:1	210–220

[a] Determined from length of plateau.
[b] Determined from extrapolation of the downcurve.
[c] Determined from chemical analysis of the insoluble material.

TABLE III.—INTERACTIONS OF VARIOUS AROMATIC ALCOHOLS AND PHENOLS WITH SARCOSINE ANHYDRIDE AT 25°

Compound	Orig. Solubility, Molar Concn. × 10²	K_1[a] (liter)/(mole)	K_2[b] (liter)/(mole)	Increase in Solubility With a 1% Sarcosine Anhydride Soln.
8-Hydroxyquinoline	0.41	8.2	1,000	59
Gallacetophenone	3.38	8.3	120	46
Phloroglucinol	16.5	12	18	29
2,5-Dihydroxybenzoic acid ethanolamide	28.6	39	10	23
2,3-Dihydroxyquinoxaline	0.15	2.5	800	18
o-Aminophenol	2.96	2.3	37	15
p-Aminophenol	5.03	1.9	18	15
m-Aminophenol	28.5	5.6	5.6	14
Salicylamide	1.82	3.9	110	26
Salicyl alcohol	59.5		2.9	13
Salicylamine	7.80	0.8	4.8	5
1,5-Naphthalenediol	0.15	23[c]		
2,7-Naphthalenediol	2.55			
Hydroquinone	63.5			
2,6-Dihydroxytoluene	212			
Hexylresorcinol				

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis.
[c] Value from downcurve is 22.

added)—(amount in soluble complex)—(amount precipitated as complex on the downcurve). When the stability constants were calculated on the basis of the stoichiometry found for the insoluble complexes, the same value for the stability constant was obtained from the rising and descending portions of the curve. The constant was not the same on both sides of the plateau if a 1:1 ratio was assumed when the plateau indicated a 2:1 complex to be the true stoichiometry. Thus, in these cases, it appears that the same reaction is responsible for the rising, descending, and plateau portions of the phase diagram. Since the 2:1 interaction is the one encountered most frequently with the insoluble complexes, it seems reasonable to assume that this is the dominant interaction responsible for many of the soluble complexes. This is especially true for the difunctional benzene derivatives, as they would be analogous to the *meta*- and *para*-hydroxybenzoic acids. The interactions between sarcosine anhydride and salicyl alcohol, orthoxylylene glycol, and *meta*-xylylene glycol also give evidence of a 2:1 stoichiometric ratio. The slopes of the straight lines obtained in these instances are all greater than 1, and it is obvious that there cannot be more sarcosine anhydride in the form of the molecular complex than has been added to the system.

In many cases, the phase diagrams for the interactions resulting in the formation of insoluble complexes exhibit a minimum in the curve at high concentrations of the complexing agent. No attempt has been made to study the interactions responsible for this irregularity, although it is believed to be a result of the formation of more soluble complexes of higher stoichiometric ratios.

The degree of solubilization of the proton-donor compounds was found to relate to the volume fraction of sarcosine anhydride in the system. It can be readily seen from Fig. 3 that the volume fraction

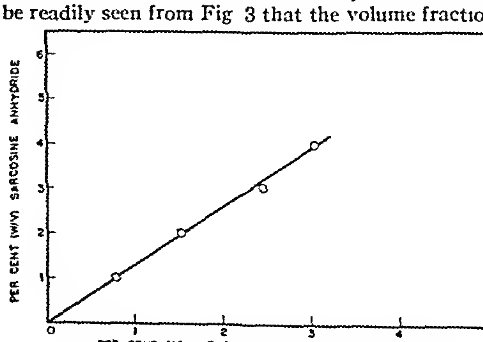

Fig. 3.—Plot showing the relationship of per cent volume fraction of sarcosine anhydride to per cent (w/v) of sarcosine anhydride.

TABLE IV —STOICHIOMETRIC RATIOS AND MELTING POINTS OF THE INSOLUBLE COMPLEXES FORMED BETWEEN AROMATIC ALCOHOLS AND PHENOLS AND SARCOSINE ANHYDRIDE

Compound	Molecular Ratio Found of Alcohol or Phenol Sarcosine Anhydride: Plateau[a]	Extrapolation[b]	Chem Anal[c]	Probable Stoichiometric Ratio	M p of Complex °C
Hydroquinone	2 21	1 9 1	2 0 1	2 1	184–185
Hexylresorcinol			4 9 1	5 1	76–77
2,6 Dihydroxytoluene	2 0 1	2 6 1	2 2 1	2 1	170–171
1,5 Naphthalenediol	1 1 1		1 1 1	1 1	214–216d
2,7-Naphthalenediol	2 9 1	2 9 1	3 0 1	3 1	171–181

[a] Determined from length of plateau
[b] Determined from extrapolation of the downcurve
[c] Determined from chemical analysis of the insoluble material

TABLE V —INTERACTIONS OF XYLYLENE GLYCOLS WITH SARCOSINE ANHYDRIDE AT 25°

Compound	Orig Solubility Molar Concn $\times 10^2$	K_1[a] (liter) (mole)	K_2[b] (liter) (mole)	Increase in Solubility With a 1% Sarcosine Anhydride Soln , %
m-Xylylene glycol	58 5		5 2	17
o Xylylene glycol	114		5 9	11
p Xylylene glycol	41 1	1 4	0 1	6

[a] Apparent stability constant calculated on a 1 1 basis
[b] Apparent stability constant calculated on a 2 1 basis

TABLE VI —INTERACTIONS OF ALIPHATIC DICARBOXYLIC ACIDS WITH SARCOSINE ANHYDRIDE AT 25°

Compound	Orig Solubility Molar Concn $\times 10^2$	K_1[a] (liter) (mole)	K_2[b] (liter) (mole)	Increase in Solubility With a 1% Sarcosine Anhydride Soln %
Suberic acid	0 82	2 2	120	12
Pimelic acid	28 5	1 4	1 9	9
Adipic acid	14 8	0 5	1 7	3

[a] Apparent stability constant calculated on a 1 1 basis
[b] Apparent stability constant calculated on a 2 1 basis

of sarcosine anhydride is a straight line function of the concentration within the concentration limits of the study Inasmuch as the solubility curves are also straight lines, it follows that the increase in solubility is related to the volume fraction of complexing agent in the same manner

The relative complexing activity taking place in these systems can also be compared by comparing the per cent increase in solubility of the acid compounds at some definite concentration of complexing agent It is interesting, however, to consider first the expected increase in solubility if there were no greater tendency for the acidic substance to associate with the complexing agent than with the solvent water If this were the case, formation of a complex would result only from a chance nonspecific contact of acid molecules with sarcosine anhydride In a 1% sarcosine anhydride solution where the volume fraction of the complexing agent is 0 008 there is a rough probability of eight chances in a thousand that a neighbor of an acid molecule would be the complexing agent Let us assume that such contacting pairs are, at the most, a separate species Since each acid molecule has, very crudely, ten neighboring molecules in the solution, this probability is increased ten times, and the expected increase in solubility would be roughly 8%, at the most, of the original solubility of the proton-donor substance Where the increase in solubility is significantly less than this value of 8%, the complex formed may be attributed to a nonspecific solvation effect as opposed to active complexing The latter must arise from some driving force, which forms complexes in solution, is substantially greater than that predicated by simple statistical expectation, and is obviously present in those interactions which give increases in solubility markedly greater than this calculated value The per cent increase in the solubility of the electrophilic compounds with a 1% sarcosine anhydride solution are included in Tables I, III, V, VI, and VII

Interactions of Sarcosine Anhydride With Various Aromatic Carboxyl Acids —This part of the study was undertaken to determine the influence of ring location on formation of molecular complexes in aqueous solution between sarcosine anhydride and various isomeric aromatic acids The interactions responsible for these complexes would be expected because of the electrophilic and nucleophilic nature of the reactants Further, the effect of various proton donating groups on the ability of these acids to form complexes was noted

The results for the interactions between a series of aromatic carboxylic acids and sarcosine anhydride are given in Table I The apparent stability constants have been evaluated and are listed under the columns headed K_1 and K_2 The values listed under the heading K_1 were calculated on the basis of a one to one interaction while those listed under K_2 were determined on the assumption of an interaction involving two molecules of the acidic substance and one of sarcosine anhydride Although the 2 1 stoichiometric ratio appears to be the one most frequently encountered in this study, it is difficult to use the apparent stability constant calculated on this basis as a sole measure of the complexing activity taking place in the system The reason for this is twofold the complex may be the result of a series of interactions and not necessarily the same in every case, and the compounds studied varied greatly in their original solubilities, and since this factor is squared in the calculation, the importance of these differences is exaggerated The relative ability of these compounds to form complexes with sarcosine anhydride, however, can be qualitatively characterized by means of the constant calculated on the assumption of a 1 1 molecular ratio, or from the per cent increase in solubility with a 1% solution

TABLE VII.—INTERACTIONS OF VARIOUS AROMATIC POLAR COMPOUNDS WITH SARCOSINE ANHYDRIDE AT 25°

Compound	Orig. Solubility Molar Concn. $\times 10^2$	$K_1{}^a$ (liter) (mole)	$K_2{}^b$ (liter) (mole)	Increase in Solubility With a 1% Sarcosine Anhydride Soln., %
Rutin	0.0055	11.3	1×10^6	91
Oxytetracycline	0.04	2.5	2,600	17
Melamine	4.20	2.0	23	13
Chloramphenicol	1.21	1.7	68	12
Quinine hydrochloride	13.3	1.4	4.9	11
Picric acid	5.50	1.5	13	10
Phenobarbital	0.55	1.8	170	9
L-Tyrosine	0.31	1.4	220	7

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis.

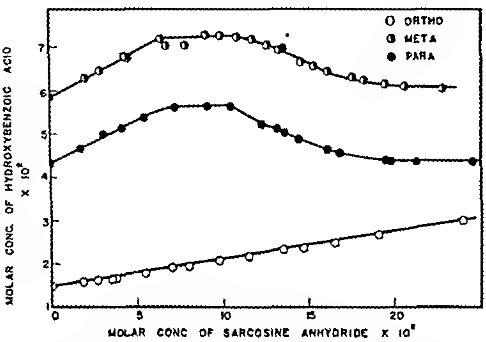

Fig. 4.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of the hydroxybenzoic acids in water at 25°. The system contained a total of 0.145 mole/L. of the *meta* and *para* isomers in and out of solution.

of the complexing agent. Although the solubility differences also influence the constants evaluated on a 1:1 basis, where the original solubilities are of the same magnitude, the effect is minor. Where a series of compounds show a wide variance in their original solubilities, the per cent increase in solubility seems to be the best method of comparing complexing activity.

It can be seen from Table I that for the hydroxybenzoic acids the *para* isomer exhibits the strongest complexing tendency, followed by the *ortho* and *meta* isomers. The phase diagrams describing these interactions are shown in Fig. 4. The apparent stability constants for the *para* and *meta* compounds were calculated on both sides of the plateau. When these constants were determined on the basis of a 1:1 ratio, the values obtained on the ascending and descending portions of the curve were not the same. If the stoichiometric ratio determined for the insoluble complex, 2:1, is utilized, however, the values for the constants do agree. This would seem to indicate a "sandwich" type structure in solution. The results for the interaction involving the aminobenzoic acids correspond to those for the hydroxybenzoic acids in that the order of activity is *para* > *artho* > *meta*.

Since in an isomeric series of compounds there is a marked difference in the ability of the various substances to enter into complex formation, the position of the polar groups, as expected, appears to be important as well as the nature of these groups. The most stable complexes involving sarcosine anhydride would be expected where the polar molecule is capable of a two-point attachment with the nucleophilic centers of the complexing agent. The molecular models show that this condition is satisfied where the polar groups on a benzene ring are *para* to one another. The results obtained for the amino and hydroxybenzoic acids agree with this observation. The *ortha* isomers in both instances appear to form, however, more stable complexes than the *meta*.

It is interesting to note that in every instance, except for the *meta*-aminobenzoic acid, the increase in solubility with a 1% sarcosine anhydride solution was substantially greater than the maximum value of 8%, predicted from statistics for nonspecific interaction. This indicates a definite specific affinity of these compounds for sarcosine anhydride.

The binding tendency which aspirin exhibits for sarcosine anhydride was found to be of the same order as that shown by benzoic acid. This is to be expected if the reactions responsible for this tendency are between the electron-rich groups of the anhydride and the electrophilic groups of the acids. On this basis, however, the complexing activity of *ortho*-phthalic acid and *ortho* and *meta*-aminobenzoic acids should be greater than the observed results indicate. The only apparent explanation for these anomalies is an increase in the solute-solute and solute-solvent interactions with these substances.

The results obtained for the 4-aminosalicylic acid interaction gives an indication that the complexing activity of a multifunctional compound is, very crudely, the sum of the activities of each of its polar groups. If the "per cent increase" values listed in Table I are considered, it can be seen that *ortho*-hydroxybenzoic acid is increased in solubility by 30%, while benzoic acid only shows a 19% increase. Similarly *para*-aminobenzoic acid has a value 7% greater than that for benzoic acid. If the complexing properties were purely additive, the increase in solubility expected with 4-aminosalicylic acid would be 37%, which agrees very well with the value, 36% obtained experimentally.

The phase diagrams for the dihydroxybenzoic acids exhibit plateau regions indicating insoluble complex formation. The phase diagrams for the interactions of 2,4- and 3,4-dihydroxybenzoic acids with sarcosine anhydride are shown in Figs. 5 and 6, respectively. It was possible, in these cases, to calculate apparent stability constants for the reaction products. The other acids in this series, however, had no rising portion on the solubility curves, which made it impossible to calculate constants

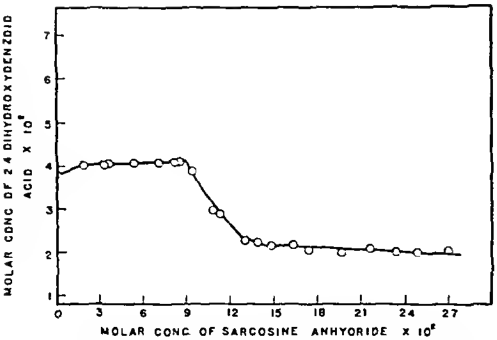

Fig 5—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 2,4-dihydroxybenzoic acid in water at 25° The system contained a total of 0 195 mole/L of 2,4-dihydroxybenzoic acid in and out of solution

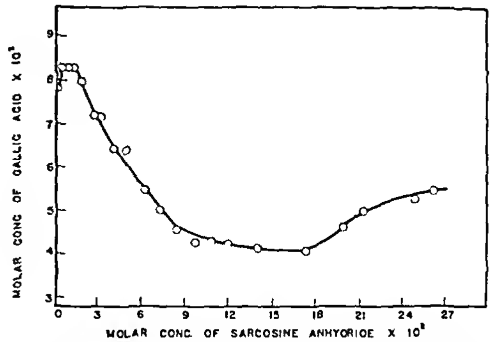

Fig 7.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of gallic acid in water at 25° The system contained a total of 0 147 mole/L of gallic acid in and out of solution

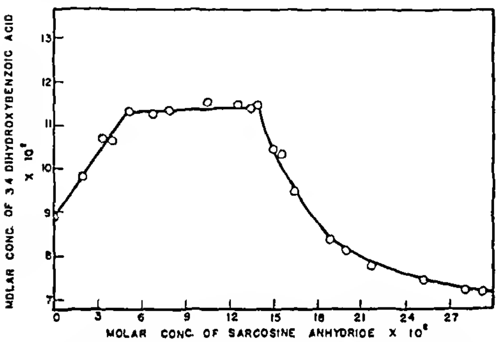

Fig 6—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 3,4-dihydroxybenzoic acid in water at 25° The system contained a total of 0 325 mole/L of 3,4-dihydroxybenzoic acid in and out of solution

for the interactions When these constants were determined on the basis of a 2 1 molecular ratio, which is the stoichiometry of the insoluble complex, there was agreement between the values obtained from the ascending and descending portions of the curve The only trihydroxybenzoic acid studied, gallic acid, also resulted in the formation of an insoluble complex and the phase diagram for this interaction is shown in Fig 7 There was a small increase in the solubility of the acid in this case, which permitted the calculation of an apparent stability constant The value of this constant, 64, determined on a 1.1 basis, indicates a strong complexing tendency of gallic acid for sarcosine anhydride The constant calculated from the stoichiometry of the insoluble complex, 6 1, was 5.5×10^5 for the upcurve and 4.7×10^5 on the downcurve A 6 1 interaction, however, would hardly seem likely in the solution phase

Table II lists the aromatic acids which formed insoluble complexes with sarcosine anhydride along with the melting points and stoichiometric ratios of the complexes A stoichiometry of two acid molecules to one molecule of sarcosine anhydride is found in the majority of insoluble complexes formed in this study Gallic acid and 3,5-dihydroxybenzoic acid, however, form insoluble addition products which have six acid molecules associated with each molecule of complexing agent The phase diagram for the interaction between 3,5-dihydroxybenzoic acid and sarcosine anhydride is presented in Fig 8

The multihydroxybenzoic acids would be expected to form stable complexes with sarcosine anhydride since in every case, except 3,5-dihydroxybenzoic acid, the polar groups are situated so as to be conducive to a two-point attachment with the nucleophilic centers of the complexing agent If the interacting molecules are joined at two points, the 2 1 complexes may result from one molecule of acid attaching to each side of the anhydride molecule to form a "sandwich" It is difficult, however, to rationalize higher stoichiometric ratios on this basis Other reasonable explanations for these complexes may be that the acidic substances exist in solution as dimers, trimers, or polymers of the interacting species, or that a 1 1 reaction forms a nucleus which then associates with other molecules of the donor substance A more detailed investigation of the insoluble materials including X-ray diffraction studies will be necessary to elucidate the nature of the interactions responsible for complexes of this type

Interactions of Sarcosine Anhydride With a Series of Aromatic Alcohols and Phenols.—Table III lists the aromatic alcohols and phenols studied, along with some of their physical properties and the results of their interactions with sarcosine anhydride The basis for choosing these compounds was to demonstrate better the effect of various polar groups and their positions on the binding tendencies of the parent molecule Since these compounds vary so greatly in their original solubilities the per cent increase in solubility with a 1% sarcosine anhydride solution appears to be the best means of comparing their complexing tendencies.

Sarcosine anhydride and 8-hydroxyquinoline appear to interact significantly with a K_1 value of 8 2 as shown in Table III The phase diagram for this interaction is illustrated in Fig 9 There are two possible explanations to account for this relatively strong interaction· (*a*) the amphoteric nature of this compound would render the 8-hydroxyquinoline–sarcosine anhydride interaction quite favorable and (*b*) the fused ring structure of the compound with its greater hydrophobic character would increase the influence of the "squeezing out" effect which has been discussed by *Higuchi* and *Lach* in a previous publication (4)

Gallacetophenone, phloroglucinol, and 2,5 dihy-

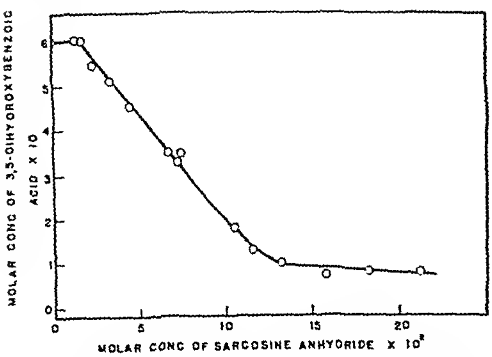

Fig. 8.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 3,5-dihydroxybenzoic acid in water at 25°. The system contained a total of 0.731 mole/L. of 3,5-dihydroxybenzoic acid in and out of solution.

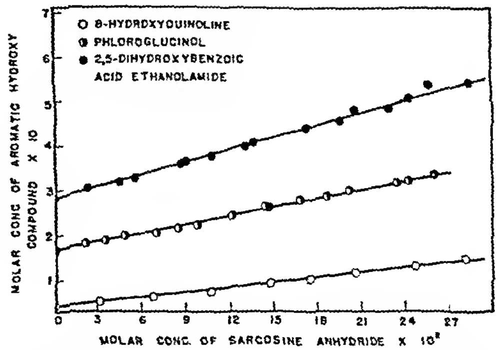

Fig. 9.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 8-hydroxyquinoline, phloroglucinol, and 2,5-dihydroxybenzoic acid ethanolamide in water at 25°.

droxybenzoic acid ethanolamide all showed relatively strong binding tendencies as would be expected with multihydroxy compounds. The phase diagrams for the interactions involving the latter two compounds are presented in Fig. 9. The comparatively strong complexing activity of 2,3-dihydroxyquinoxaline may be attributed to the highly dipolar nature of this fused ring compound.

The order of activity for the aminophenol isomers would be expected to be the same as that exhibited by the aminobenzoic acids. This was not the case, as the *ortho*-aminophenol showed greater activity than the *para* compound which in turn was more active than the *meta* isomer. It was interesting to note, that for this series of compounds the complexing activity and solubility seem to be related, i. e., the least soluble compound shows the greatest activity and the most soluble compound shows the least activity.

The relative binding tendencies of salicylamide, salicyl alcohol, and salicylamine were in the order expected. The molecular models show that a two-point attachment with the electron-rich centers of the complexing agent is possible with all three of these compounds. The N-hydrogen of the amide, however, would form this association more easily than the aliphatic alcohol or amine group. The relative inactivity of the salicylamine can be attributed to the ease with which intramolecular associations can occur in this molecule. The increase in solubility, 5% with a 1% sarcosine anhydride solution is also less than 8%, the maximum expected on a nonspecific, statistical basis, indicating the relatively weak affinity of this compound for the complexing agent. The slope of the solubility curve describing the interaction between salicyl alcohol and sarcosine anhydride is greater than one. Therefore, it appears that the reaction product has more than one molecule of the alcohol associated with each molecule of complexing agent; otherwise the results indicate that more sarcosine anhydride is present in the form of the complex than was added to the system.

The stoichiometric ratios and melting points of the insoluble complexes formed between sarcosine anhydride and the aromatic hydroxy compounds are listed in Table IV. It can be seen from the phase diagrams illustrated in Figs. 10 and 11 that the 1,5- and 2,7-naphthalenediol isomers both formed

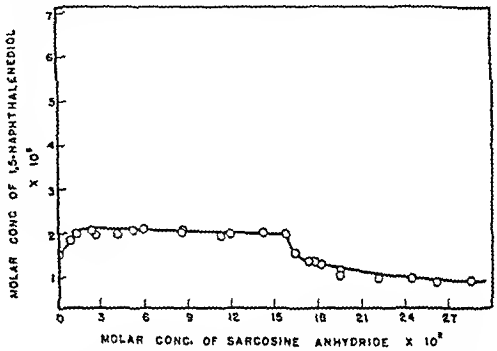

Fig. 10.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 1,5-naphthalenediol in water at 25°. The system contained 0.156 mole/L. of 1,5-naphthalenediol in and out of solution.

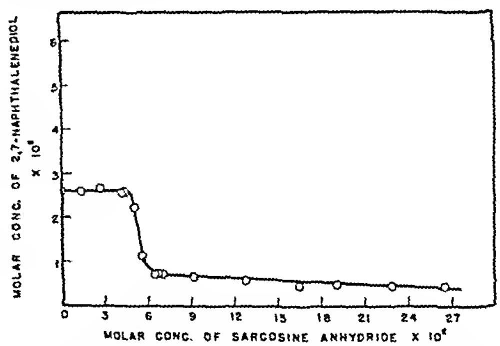

Fig. 11.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 2,7-naphthalenediol in water at 25°. The system contained 0.156 mole/L. of 2,7-naphthalenediol in and out of solution.

insoluble reaction products. In the case of the 1,5 compound there was an initial rise in the solubility curve which permitted the calculation of an apparent stability constant. The stoichiometric ratio of the insoluble material was 1:1, consequently, the constant was determined on this basis. The values obtained from the portions of the curve with a positive and negative slope were in agreement indicating that the principal reaction occurring is of a 1:1 nature. The value of the constant, 23, indicative of a strong binding tendency. A stal

complex would be expected with this compound in view of its fused ring system which would increase the influence of the "squeezing out" effect and the position of the polar groups which make possible a two point attachment with the complexing agent The 2,7-naphthalenediol resulted in an insoluble complex with a stoichiometric ratio of 3 1 The absence of an initial increase in the solubility of the compound made it impossible to calculate a stability constant for the complex

The interactions involving hydroquinone, 2,6-dihydroxytoluene and hexylresorcinol all resulted in the formation of insoluble complexes The phase diagram representing the hydroquinone-sarcosine anhydride interaction is illustrated in Fig 12 and shows that the insoluble complex is precipitated immediately A similar solubility curve was obtained for the 2,6 dihydroxytoluene and sarcosine anhydride interaction, therefore, the apparent stability constants of these reaction products could not be determined A phase diagram for the hexylresorcinol interaction could not be obtained because the excess hexylresorcinol in the system formed an emulsion which made it impossible to obtain a sample of the solution for analysis By using an excess of sarcosine anhydride it was possible, however, to precipitate an insoluble complex which was found by chemical analysis to have a 5 1 stoichiometric ratio

Hydroquinone may be expected to associate strongly with sarcosine anhydride because of the favorable *para* relationship of the hydroxy groups on the benzene ring Whether this is the case is difficult to establish because of the extreme insolubility of the complex, evidenced by the steep slope of the descending portion of the phase diagram and lack of any detectable initial increase in solubility with this compound

The variety of stoichiometric ratios exhibited in this study may be rationalized on the basis discussed previously for the insoluble products which resulted from interactions between aromatic acids and sarcosine anhydride

Interactions of Sarcosine Anhydride With the Xylylene Glycols.—The xylylene glycols were studied to determine the possible steric effects on interactions with sarcosine anhydride From observations of the molecular models it would appear that the configuration of the *ortho* and *meta* isomers are

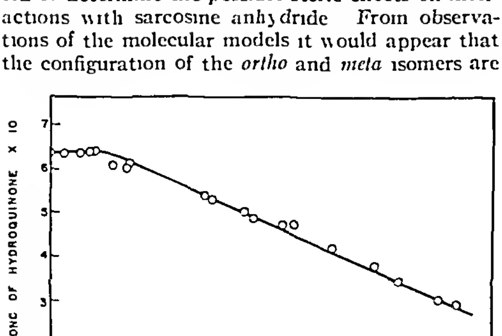

Fig 12—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of hydroquinone in water at 25° The system contained 0 727 mole/L of hydroquinone in and out of solution

suited for a two-point attachment with the complexing agent It can be seen from Table V, however, that the order of complexing activity was *meta* > *ortho* > *para* The reactivity of the *meta* isomer may be magnified in this study because of the use of a 20% sodium chloride solution as the solvent which was used to decrease the initial solubility of this compound This would mean that the ratio of the volume fraction of sarcosine anhydride to the volume fraction of water would be greater in this system than in the other Therefore, the relatively strong binding tendency exhibited by the *meta* isomer may be influenced by an increase in the non specific solvation effect

The solubility curves for both the *ortho* and *meta* xylylene glycols have slopes greater than one, indicating a stoichiometric ratio higher than 1 1 for these complexes The *para* isomer showed a low degree of activity as would be expected for a compound of this type where the steric requirements were unfavorable and the polar groups are removed from the aromatic nucleus

Interactions of Sarcosine Anhydride With Aliphatic Dicarboxylic Acids.—A series of aliphatic dicarboxylic acids were included in the study in order to show the influence of an aromatic structure on interactions of this type Table VI lists the acids used in this study along with the results of the interactions and some physical properties of these aliphatic compounds The solubility curves for these compounds demonstrate the weak interactions between sarcosine anhydride and nonaromatic acids Since the carboxy groups of pimelic and suberic acids are at the proper distance for a two point attachment with the sarcosine anhydride molecule, it appears, from the weak binding tendencies exhibited by these compounds, that the presence of an aromatic structure is essential for the donor substance to show a strong attraction for the complexing agent, sarcosine anhydride

It is interesting to note that the complexing tendencies of these acids appear to be related to the chain length of the compounds in that the longer the carbon chain, the greater the tendency to complex This may be due to the increased hydrophobic portion of the longer chained molecules and its resultant influence on the "squeezing out" effect

The wide differences in the water solubilities of these substances may also play an important role in the relative reactivities of these aliphatic acids For example, the high water solubility of pimelic acid would indicate a strong solute solvent interaction which in turn would decrease the tendency of this compound to form a complex with sarcosine anhydride

Interactions of Sarcosine Anhydride With Various Other Polar Compounds.—A group of miscellaneous compounds which were included in this study are listed in Table VII along with the results of their interactions with sarcosine anhydride and some of their physical properties

Solutions of sarcosine anhydride have been used to solubilize rutin and similar compounds in several commercial preparations in order to make concentrated solutions of these substances to be used as injectables The results of this study indicate that the solubilization of these substances may be the result of molecular complex formation

The very strong complexing activity exhibited by

rutin is not unexpected when the aromatic character and abundance of phenolic hydroxy groups on the molecule are considered. These same factors are important in the interaction of oxytetracycline and sarcosine anhydride. The large difference in complexing activity between rutin and oxytetracycline can be attributed to the ease with which intramolecular hydrogen bonds can form in the oxytetracycline molecule

Melamine gave results which were somewhat higher than expected in view of the fact that the molecule has no carboxylic acid or hydroxy group. These groups appear to have the greatest effect on the complexing tendencies of the compounds studied.

The binding tendency exhibited by chloramphenicol fits in nicely with the results obtained with the xylylene glycols. In both cases the degree of complexing can be considered to be influenced by the alcohol groups and aromatic nucleus. The quinine hydrochloride molecule also resembles these previously mentioned alcohols, differing in that it has only one hydroxy group but a larger aromatic nucleus and greater hydrophobic character. Thus, the degree of complexing activity exhibited by quinine hydrochloride is consistent with the results obtained for compounds with similar structures.

The relatively weak interactions between picric acid, phenobarbital, and L-tyrosine with sarcosine anhydride were not entirely unexpected. In the interaction involving picric acid, the presence of the nitro groups on the molecule may interfere with the ability on the phenolic hydroxy to associate with the complexing agent. While in the case of the barbituric acid derivative, none of the polar groups which appear to be necessary for strong binding with sarcosine anhydride are present. The inactivity of L-tyrosine can be ascribed to the intra- and intermolecular associations which are prevalent in this compound.

DISCUSSION

The results of this investigation give definite evidence for the formation of both soluble and insoluble molecular complexes in aqueous solution between sarcosine anhydride and various proton-donor compounds. There is also an indication that the nature of the electrophilic groups and their position on the molecule influence the degree of complexation. Furthermore, the weak binding tendency encountered with the aliphatic dicarboxylic acids point out the importance of an aromatic system in these interactions. For a molecule to show optimum complexing activity with sarcosine anhydride, it appears that it should have an aromatic nucleus, one or more acid or hydroxy groups attached to the aromatic structure, and the electrophilic groups should be in the optimum position to make a two-point attachment with the nucleophilic centers of the complexing agent.

A comparison of the complexing tendencies of the acidic compounds indicates that the compounds with greater hydrophobic character show greater complexing activity than the more soluble substances This correlation can be attributed to the increased influence of the "squeezing out" effect with the less soluble materials and the stronger solute-solvent interactions encountered with the more water-soluble donor compounds Even with comparatively soluble compounds, however, relatively stable complexes can be formed if the steric relationships are favorable

The stability constants for the complexes formed with sarcosine anhydride cannot be compared with the constants for the xanthine complexes, since with the anhydride there is evidence to indicate that interactions other than the 1:1 type are occurring simultaneously The stability constants obtained by Higuchi and Kostenbauder (10), however, for the complexes formed between a series of water-soluble amides such as the N, N, N′, N′-tetramethyl derivatives of phthalamide, isophthalamide, terephthalamide, fumaramide, and succinamide; and chloramphenicol, *para*-hydroxybenzoic acid, aud salicylic acid are of the same magnitude as the constants obtained in this study with the same donor compounds It would appear reasonable, therefore, to expect the same type interactions to be responsible for the complexes with the substituted cyclic amide, sarcosine anhydride, as in the case of the previously mentioned water-soluble amides.

These findings lend further credence to the belief that interactions of this nature are important in mediating many enzymatic and drug actions Since the diketopiperazine structure with its cyclic dipeptide linkage grossly resembles a segment of a protein alpha helix, the type of binding experienced for this compound is probably representative of those utilized by the biochemical macromolecules. The significant tendency of simple oxide groupings to form molecular complexes with a large array of organic compounds as shown in these studies, it is apparent, cannot be ignored in any detailed formulation of mechanism of *in vivo* actions

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Further Studies on the Effects of Ephedrine in the Presence of Cocaine*

By PHILLIP V. HAMMOND

It has been demonstrated that cocaine will lessen some of the effects of ephedrine, chiefly cardiac output and blood pressure changes. The nature of the finer mechanism of action of these changes has not been ascertained. Because of the many unrelated sites of action it is believed there may possibly be more than one type of mechanism of action involved. Diminution of response to cocaine is largely cardiac in nature because vasoconstriction and increase in peripheral resistance in the femoral vascular bed was not marked. This action is chiefly central in origin because it cannot be demonstrated on the isolated rabbit heart. This indicates that the theory of Gaddum and Kwiatkowski (9) that the action of ephedrine is mediated chiefly through the presence of epinephrine cannot be demonstrated here, since cocaine will potentiate the action of epinephrine on the isolated rabbit heart. It is believed that the nature of the antagonism between ephedrine and cocaine is noncompetitive, e. g., the two drugs combine with different parts of the receptor mechanism.

EPHEDRINE, an important and useful drug, bears many striking pharmacological resemblances to epinephrine; however, there exist several significant differences, such as the action of the two drugs *in vivo* in the presence of cocaine. We have found, as Tainter (1), Holck (2), and other investigators (3), that some of the effects of ephedrine in the cocainized animal are either greatly reduced or abolished

Besides being of academic interest, it was felt that a further investigation of this phenomenon might cast additional light on the somewhat nebulous mechanism of action of ephedrine, and possibly elucidate the site or sites of action With this in mind, it is the purpose of this paper to study the relationship of ephedrine to cocaine with special reference to the cardiovascular system.

EXPERIMENTAL

Pressor Response.—Twenty-four mongrel dogs were used throughout the pressor and related experiments

In conducting these experiments, an acute mammalian set-up was utilized. The procedure was to ascertain the normal blood pressure after the animal had become stabilized under pentobarbital sodium anesthesia (30 mg /Kg). Ephedrine 0 3 mg /Kg was administered intravenously, this was followed by cocaine administered subcutaneously in varying doses ranging from 4 mg /Kg. to 30 mg /Kg After the animal had become cocainized (twelve to fifteen minutes) a second dose of ephedrine was given This procedure was repeated three or four times in each experiment In some of the experiments the animal was cocainized without a previous *injection of ephedrine, and after the proper time interval* ephedrine was administered. Pressure responses to ephedrine were also obtained from the femoral artery.

By setting up experiments in which ephedrine was administered at varying time intervals in the absence of cocaine it was determined that tachyphylaxis was not a factor in the diminution in response to the pressor action of ephedrine. Tachyphylaxis was ruled out when, after a suitable time interval, an injection of ephedrine produced the same pressor response as the original dose. Electrocardiographic changes were taken with a Sanborn Viso-Cardiette using standard limb leads

Isolated Heart.—The action of ephedrine in the presence of cocaine was determined on the isolated rabbit heart using the Langendorff and Anderson (4) perfusion pump The animal was sacrificed The heart was quickly removed and placed into the heart chamber Here it was perfused with an oxygenated, modified Ringer's solution at a constant temperature of 37 5°. After the steady amplitude and the steady rate of contraction had been determined, the two drugs were perfused through the preparation at definite intervals Cocaine was used in doses ranging from 0 12 mg to 0 25 mg and ephedrine was used in doses ranging from 0 1 mg to 1 32 mg

Action on Vascular Bed.—The action of ephedrine on the vascular bed in the cocainized animal was tested by anesthetizing dogs in the manner previously mentioned Five hundred U. S P. units of heparin or 250 mg /cc. of mepesulfate[1] were administered Throughout these experiments heparin, mepesulfate, or pontamine Fast Pink BL standard[2] were given when necessary as anticoagulants Both femoral arteries were isolated and cannulated Blood pressure readings were taken from the right femoral artery, whereas the arterial flow into the left vascular bed was measured from the left femoral artery by means of a Shipley and Wilson (5) rotameter Changes produced were visual Desirable changes were recorded by means of a

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Preliminary reports on certain types of the experiments herein described appeared in THIS JOURNAL, 47, 67(1958)

[1] Hoffmann LaRoche—Formerly known as Treburon

[2] E I du Pont de Nemours and Co , Inc

Sanborn Viso-Cardiette Model 572 M. A 500-cc. polyethylene bottle was used in the system as a dampening chamber. At the end of the experiments the rotameter was calibrated using a graduated cylinder and stop watch. In one experiment an eight-channel recorder was used to record aortic pressure, volume, and flow. Cocaine in doses ranging from 18–22 mg./Kg. was administered subcutaneously, whereas ephedrine in doses of 0.3 mg./Kg. was administered into the system by injecting it into a plastic chamber attached to a magnetic stirrer. A total of six dogs was used.

Visceral Blood Vessels.—The action of the two drugs was tested on visceral blood vessels by means of an acute mammalian set-up. The carotid artery was isolated and cannulated. The femoral vein was isolated and used as a site for the administration of ephedrine, whereas cocaine was administered subcutaneously. A portion of the small intestine was exposed and placed in an oncometer. Readings were made by means of kymograph.

Cardiac Output.—Cardiac output studies were continued by means of the radioactive isotope dilution method described by MacIntyre (6) and others, using iodinated (I^{131}) human serum albumin. Prior to the anesthetization of the dog, Lugol's solution was administered orally in order to prevent the thyroid from picking up the radioactive iodine. Pentobarbital sodium was used as the anesthetic agent. The jugular vein and femoral artery were isolated, and cannulas and Cournand needles were inserted. Iodinated (I^{131}) human serum albumin was employed in doses of 30 to 100 μc. The dilution technique employed was to dilute 1 cc. of radioactive iodinated human serum albumin to 5 cc. with normal saline. Of this dilution, 0.1 cc. was diluted to 10 cc. One cubic centimeter of the aliquot was used as a control. The volume of the dose was 4.4 cc. The dilution curve was obtained by plotting the radioactivity of the blood flowing over the detector as a function of time.

Oxygen Uptake.—In an initial attempt to determine the mechanism of action of ephedrine in the cocainized animal, oxygen uptake studies employing the Warburg respirometer were undertaken. Male mice weighing 16–18 Gm. (Swiss strain) were used in these experiments. Cocaine was administered to the intact animal in doses of 40 mg./Kg. and ephedrine in deses of 2 mg./Kg. (Controls were administered approximately the same volume of saline as the treated mice.) After sacrificing the animals, the livers were rapidly removed and placed on ice. One hundred milligrams of sample per cc. of homogenate was used. The liver samples were homogenized in Tyrode solution to which 1% of glucose had been added. The tissues were then placed in Warburg flasks which were placed in a constant temperature bath of 37°. The flasks were equilibrated for ten minutes. Uptake of oxygen was measured in ten-minute periods for two hours. One dozen animals were used in these experiments.

RESULTS AND DISCUSSION

Pressor Response.—Throughout all of the pressor experiments the effects of ephedrine on the blood pressure of the cocainized animal were greatly reduced. As seen in Fig. 1, the diminution in response

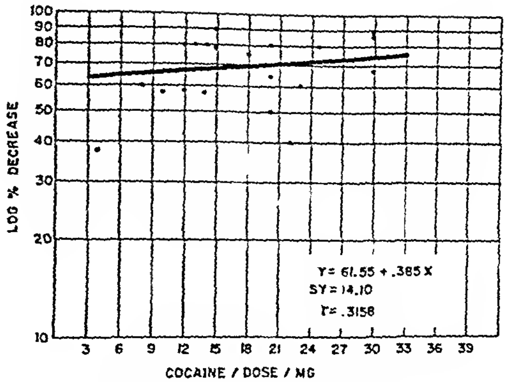

Fig. 1.—Diminution of pressor response to ephedrine in the cocainized animal.

to ephedrine was proportional to the dose of cocaine employed. For example, doses of 30 mg./Kg. of cocaine prevented 90% of the pressor response to ephedrine, whereas a dose of 10 mg./Kg. prevented 57% of the pressor response to ephedrine.

The response to ephedrine was found to be reduced following the administration of cocaine, when compared to the effects of the same dose of ephedrine given either before the injection of cocaine or after the effects of cocaine had worn off.

From the results obtained, the equation for the line was found to be $Y = 61.55 + 0.385X$. The standard error of estimate $Xy = 14.10$ and the correlation of index $Y = .3158$. Although the rho obtained may be indicative of an immoderate degree of correlation, it should be recognized that such experiments as those herein described often include biological functions which cannot be rigidly controlled and their combined influence on one or the other of the variables is difficult to determine. It is of importance, however, to note that the rho is such that it shows a trend toward a positive association between these variables.

In conducting the studies on tachyphylaxis and electrocardiographic changes the results obtained confirmed those findings previously reported by this laboratory (7), e. g., tachyphylaxis may be ruled out if sixty-five to seventy minutes are permitted between injections of ephedrine, and, whereas these drugs alone and combined produced the same significant electrocardiographic changes as previously reported, we could not make any definite conclusions from these changes alone.

Isolated Heart.—The results of 12 experiments revealed that the diminution in response to ephedrine cannot be satisfactorily demonstrated on the isolated rabbit heart. Both drugs increased the amplitude and rate of contraction until toxic levels were reached. In the nontoxic doses employed, the response to ephedrine was not lessened by the previous administration of cocaine.

Vascular Bed.—In Table I, both ephedrine and cocaine singularly produced vasoconstriction in the femoral vascular bed and an increase in peripheral resistance. The overall action of ephedrine in the presence of cocaine on the femoral vascular bed is largely additive rather than antagonistic to cocaine.

Visceral Blood Vessels.—The results of the testing of the two drugs on the visceral blood vessels, e. g., a portion of the small intestine, revealed the

TABLE I —EFFECTS OF EPHEDRINE COCAINE ON THE FEMORAL VASCULAR BED OF THE DOG

Expt No	Dose Cocaine mg /Kg	Normal P/F	Normal R	Ephedrine 3 mg /Kg P/F	Ephedrine R	Cocaine RP/F	Cocaine R	Ephedrine and Cocaine P/F	Ephedrine and Cocaine R
1	22	110/35	3.1	115/31.25	3.7	115/27	4.3	95/22.5	4.2
2	22	110/35	3.1	115/31.25	3.7	86/32.5	2.5	89/26.3	3.4
3	20	110/15	7.3	122/6.25	19.5	110/3.75	29.3	107/3.75	28.6
4	20	11/15	7.3	122/6.25	19.5	92/8.75	10.5	86/6.25	12.2
5	18	93/7.5	12.4	95/6.25	15.2	89/5	17.8	88/5	17.6
6	18	93/7.5	12.4	95/6.25	15.2	94/6.00	15.8	94/6.25	15.0

P = Mean arterial pressure F = Flow R = Resistance

average changes taken from four experiments See Table II

From these results we may conclude that ephedrine in doses of 18–20 mg /Kg does not produce any significant changes on the visceral blood vessels of the intestines

Cardiac Output.—In Table III ephedrine produced a substantial increase in cardiac output This effect was lessened by the action of cocaine No adequate explanation is being offered to explain the variation in total peripheral resistance Perhaps some of these variations were due to a compensatory action of the body This increase in cardiac output, which was later modified by cocaine, was due mainly to an increase in stroke volume

Oxygen Uptake Studies—As a means of determining if oxygen utilization was involved in the mechanism of action of ephedrine in the cocainized animal, studies on oxygen uptake were conducted on the Warburg respirometer In Fig 2 these studies revealed that during the first hour liver homogenates from animals that had been previously treated with ephedrine gave off oxygen up to 18.63%, and that liver homogenates from animals that had been treated with cocaine took up a small amount of oxygen (a maximum of 8.27%), whereas liver homogenated from animals that had been treated with ephedrine and cocaine took up a maximum of 33.08% of oxygen during the second hour It is believed that oxygen utilization will not explain the mechanism of action of ephedrine in the cocainized animals and that other enzymatic systems should be investigated According to Axelrod (8) ephedrine is demethylated by an enzyme system in rabbit liver microsomes to norephedrine and formaldehyde It is believed by this author that the giving off of oxygen during the first hour might possibly be due to formaldehyde poisoning of an enzymatic system and that this action is overcome in time, e g, within two hours, by the presence of norephedrine, enabling the liver homogenates to take up increasing amounts of oxygen and that this reaction is not affected by the presence of cocaine This is a theory that needs further investigation, for at present there is not satisfactory scientific evidence to support it

TABLE II —ACTION OF EPHEDRINE-COCAINE ON VISCERAL BLOOD VESSELS

	Normal	Ephedrine 0.3 mg / Kg	Cocaine 20 mg / Kg	Ephedrine and Cocaine
Blood pressure, mm / Hg	136	170	120	122
Ht of contraction of intestines, mm	10.8	10.6	10.6	10.6

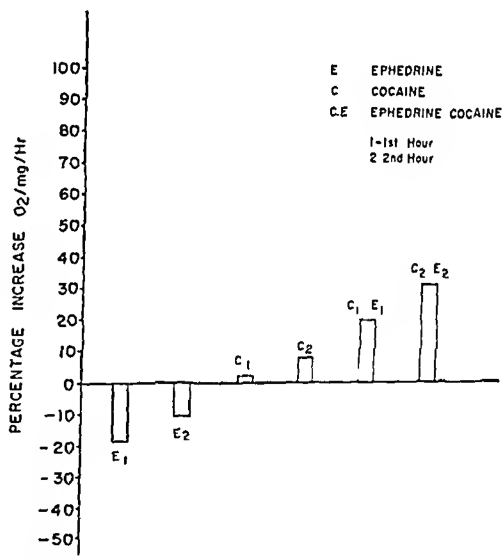

Fig 2 —Oxygen uptake, mouse liver homogenate

SUMMARY

1 The pressor response to a dose of 0.38 mg / Kg is markedly reduced in the presence of cocaine Under properly controlled conditions, this reduction amounts to 90 per cent or more

TABLE III —CARDIAC OUTPUT

	Normal H1	Normal H2	Ephedrine[a] H1	Ephedrine[a] H2	Ephedrine[a] in the Cocainized Animal H1	Ephedrine[a] in the Cocainized Animal H2
Cardiac output, l /min	3.63	1.7	5.77	2.4	5.33	1.5
Cardiac index, l /min /M²	4.5	3.5	7.2	5.2	6.67	3.3
Total peripheral resistance, dynes/cm ²	2,564	1,328	1,695	1,328	1,738	3,111
Stroke volume, cc /beat	21.4	10	32	15	30.5	9

[a] 0.3 mg /Kg

Diminution in response to the pressor effects of ephedrine in the cocainized animal is not related to tachyphylaxis.

2 The mechanism of action of the reduction to ephedrine cannot be explained on the basis of differences in electrical potentials taken from electrocardiographic changes

3 The reduction in response to ephedrine in the presence of cocaine cannot be demonstrated on the isolated rabbit heart

4 Both ephedrine and cocaine singularly acted on the femoral vascular bed to produce some degree of vasoconstriction and a decrease in blood flow However, in the presence of cocaine, the vasoconstriction and decrease in blood flow is augmented rather than decreased

5 Ephedrine and cocaine do not produce any significant action on visceral blood vessels

6 The increase in cardiac output induced by ephedrine is greatly lessened by cocaine.

7 Differences in oxygen uptake of ephedrine and ephedrine in the presence of cocaine in liver homogenates could not satisfactorily explain the diminution in the cardiac output induced by cocaine

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Drug Modification of Runway Behavior of Mice Influenced by Aversive Stimulus*

By J. A. HUNTZINGER†, J. J. WITOSLAWSKI, and H. M. HANSON

Chlorpromazine administered intraperitoneally to white mice significantly increases running time on a short runway; this effect is proportional within the range studied with increasing dosage. Following exposure to an aversive stimulus, untreated mice or mice receiving the 2 mg./Kg. of chlorpromazine markedly increased running times as a result of such stimulation. However, mice receiving the 4 mg./Kg. dose showed no increase in running time. Assuming that such increases in running time are free of interaction with the preceding level of performance and are comparable in terms of absolute differences between groups it would appear that the compound either prevented the setting up of some condition ("fear") resulting from the light stimulus, which increased the running time in the untreated mice, or it prevented the operation of the condition set up by such aversive stimulation.

THIS STUDY investigated the possibility that one of the traditional techniques for the study of instrumental behavior might be useful as a rapid first evaluation of unknown compounds believed to have psychotropic activity A precursor of such an application was Brady's study of the effects of tetraethylammonium on the runway behavior of rats (1) It was felt that by a modification of the basic method, a response to so-called "fear"-producing stimuli might become measurable. Chlorpromazine hydrochloride, 10-(γ-dimethylaminopropyl) - 2 - chlorophenothiazine hydrochloride, was selected for testing as a reference agent for drugs of the "tranquilizer" class

METHOD

Eighty male albino mice (Merck Sharp & Dohme strain) sustained on a twenty-two-hour food deprivation schedule were trained to run a 24-inch elevated runway

A timing zone of 17 inches was marked off on the runway equidistant from each end, the remaining space being designated starting and goal areas The mice were reinforced by being allowed to eat for approximately three seconds from a supply of wet mash placed at the far end of the goal area After a day's experimental session, the mice were fed wet mash *ad libitum* for one hour, water always being available

After 25 training trials, the mice were randomly divided into four groups On the next experimental day, after two trial runs, chlorpromazine was administered intraperitoneally to each group at the following dose levels: Control (saline), 2 mg /Kg , 4 mg /Kg , and 6 mg /Kg Forty-five minutes postinjection, 4 trials were run Running times were measured on the last preinjection trial and on the second and fourth postinjection trials The time required to complete a run between the two 17-inch markers was recorded in tenths of a second At the end of the third run while the mouse was still in the goal area and consuming the mash, a photoflood lamp placed approximately six inches above and in front of the mouse was

* Received June 25 1959, from The Merck Institute for Therapeutic Research West Point, Pa

† Research performed during tenure as 1957 Merck Sharp & Dohme Research Laboratories Division Industrial Fellowship for Science Teachers Present address Howard University, Washington, D C

illuminated for one second This stimulus produced freezing and/or withdrawal in all mice On the basis of earlier studies with rats it can be considered aversive, i e, it would probably be sufficient to support instrumental escape behavior (2, 3) The mouse was then immediately returned to the starting area and the fourth and final trial given If the mouse had not completed the run in sixty seconds, the trial was terminated by removing the mouse from the runway, a score of sixty seconds being recorded

RESULTS

The results are shown in Fig 1 The running times measured in tenths of a second are plotted by group averages against the dose levels tested The dashed line shows the mean preinjection running times for each group There were no significant differences (tested by Kruskal Wallis one way analysis of variance (4)) at the 0 05 level for the mean preinjection running times for the 4 groups Mean running times forty five minutes after the injection of chlorpromazine are shown in the postinjection curve An orderly increase in running times was obtained with increasing drug dosage The differences between the group means were significantly different at the 0 001 level of confidence However, differences between pre and post injection runs for the 0 and 2 mg /Kg groups, tested separately by Mann-Whitney U test, were not significantly different at the 0 05 level (4) The third curve in Fig 1 labeled 'poststimulus," shows the effect of the presentation of the brilliant light upon running times, and the interaction of this effect with the drug action In the saline control group and the 2 mg /Kg drug group the mean running times on the trial following the light stimulus were significantly different from the mean postinjection running times, at better than the 0 05 level of confidence (This increase supports the notion that the light was aversive) The 4 mg / Kg dose did not significantly increase the mean running time when compared to the postinjection trial suggesting an interaction of the two variables stimulus presentation and drug The poststimulus curve is flatter than the postinjection curve, with intersection of the two curves near the 4 mg /Kg dose value suggesting an inverse relationship with dose

The poststimulus mean running time for the 6 mg /Kg group indicates a *marked decrease* in running times following the presentation of the light stimulus and was found to be significantly different from the postinjection mean running time However, there was a decrease in the size of this group (to N = 12) by the time of the stimulation trial due to the heavy sedation produced by the injected compound Considering that probably only those animals most resistant to the effects of chlorpromazine were performing at the time of testing, it is possible that those animals might

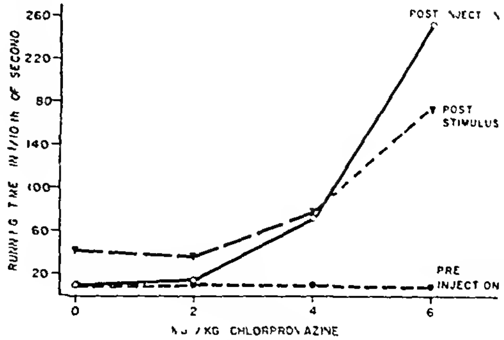

Fig 1—Average running times on an elevated runway for four groups of mice Each point represents the average group performance on a single trial Following a control trial (curve labeled "pre injection") chlorpromazine was administered at the doses indicated on the abscissa The effects of the drug are shown in the curve labeled "postinjection " The results of a final trial immediately preceded by exposure to an aversive stimulus are shown in the third curve The 6 mg /Kg point in this curve due to reduction in group size can only be considered a suggestive value

also be resistant to the effects of the stimulation For these reasons this value is probably not germane to a discussion of the results of this study

DISCUSSION

The particular experimental situation utilized for this study allows an approach to a condition more comparable to "emotion" in humans than most others generally in use in small animal experimentation The response to repeated application of aversive stimulation in rodents rapidly changes with the replication of such stimulation The first few times shock or other aversive stimulation is applied, gross effects considered as concomitants of "emotion" such as defecation, urination, pilo erection, respiratory changes, hyperactivity, and/ or "freezing behavior" are observed After a number of applications of such stimulation most of these signs are completely absent, suggesting that as an "analogue" of "emotion" the response to chronic aversive stimulation is questionable Conceivably by presenting the aversive stimulus only once and measuring the response to such stimulation immediately thereafter, as was done in the present study, a state closer to "true emotion" is made available for study

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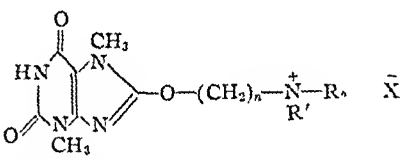
Quaternary Ammonium Salts of 8-(Dialkylaminoalkoxy)-theobromines and -caffeines as Curariform Agents*

By DIPTISH C. CHAKRAVARTY and JAMES W. JONES

Three 8-(dialkylaminoalkoxy)-theobromine compounds were prepared by reacting the appropriate sodium dialkylamino alcoholate with 8-chlorotheobromine. Nine quaternary salts were prepared from the above three compounds by reacting each with methyl iodide, ethyl iodide, and benzyl chloride. The nine quaternary compounds along with nine corresponding caffeine quaternary compounds, previously reported, were tested for their curariform activity.

Contemporary studies with synthetic curariform compounds began with Bovet's working from a model of (+)-tubocurarine (1). This was followed by the synthesis and testing of a large number of quaternary ammonium compounds. Choline derivatives were among these (2). Included among the curariform agents studied by Bovet was tris-(β-triethylammonium-ethoxy)-1,2,3-benzene triiodide (Flaxedil), which was found to be most active (3). The present study was instituted with the view of preparing a group of compounds related to phenylcholine ethers and carrying out a very preliminary testing for possible curariform activity. Consequently a number of aminoalkyl ethers and their quaternary ammonium salts having the following general formula have been prepared from 8 chlorotheobromine.



The present syntheses represent an extension of the work with the corresponding derivatives of caffeine (4).

EXPERIMENTAL

8-Chlorotheobromine.—This was prepared according to Biltz and Beck (5). Fifty grams of theobromine, suspended in 500 ml. of dry chloroform was refluxed at room temperature while a stream of dried chlorine was bubbled through it for six hours. The 8 chlorotheobromine was separated from the reaction mixture and recrystallized from glacial acetic acid. Yield—98%, m. p. 297°.

8 - (Dialkylaminoalkoxy) - theobromines.—The sodium amino alkoxides were prepared in a 500 ml., three necked flask fitted for refluxing by allowing 2.6 Gm. (0.12 mole) of sodium metal, cut into small pieces, to react with 0.18 mole of the appropriate alcohol (dimethylaminoethanol, diethylaminoethanol, or diethylaminopropanol) in 50 ml. of dry benzene. The mixture was warmed over a water bath to complete the reaction as indicated by the absence of sodium particles. 8-Chlorotheobromine (0.12 mole) was added and refluxing with stirring was continued for two hours, by which time the reaction was complete. The solution was filtered while hot. The filtrate was transferred to a separatory funnel, washed three times with 50 ml. portions of water, dried over anhydrous sodium sulfate, and refiltered. Benzene was then removed under reduced pressure. Another 50 ml. portion of benzene was added and subsequently removed as above. The damp material was dried in an oven at 60° and recrystallized from benzene solution. The melting points and analyses are shown in Table I.

Quaternary Salts of 8-(Dialkylaminoalkoxy)-theobromines.—The aminoalkyl ether (0.01 mole) was dissolved in 50 ml. of absolute ethanol, and 0.015 mole of methyl iodide, ethyl iodide, or benzyl chloride was added with thorough stirring. The solution was placed in a refrigerator until the quaternary salt crystallized out. The salt was filtered out while cold, redissolved in absolute ethanol, and precipitated with ether. This process was repeated for each salt until a constant melting point was obtained. The melting points, analyses, and yields of the quaternary salts are given in Table II.

Pharmacological Studies.—These studies were only preliminary in scope to determine whether or not the compounds exhibited sufficient curariform activity to justify a complete activity study. They were carried out by the method used primarily for testing the curariform activity. A modification of the screen-drop test described by Cavallito, *et al.* (6), and the head drop test (7) were used to determine the relative potencies of the compounds. In the mouse screen drop test, groups of twelve white mice were used at each dosage level. The ED_{50} was determined using the method of probit analysis described by Burn (8). The agents were administered intraperitoneally in 0.25 ml. of solution.

TABLE I.—8-(DIALKYLAMINOALKOXY)-THEOBROMINES

R	n	M. p.	Yield %	—Nitrogen %— Calcd.	Found
CH_3	2	149–150	26	26.2	26.1
CH_3CH_2	2	172–173	25	23.7	23.7
CH_3CH_2	3	161–162	23	22.6	22.6

* Received March 26, 1959, from the State University of Iowa, College of Pharmacy, Iowa City.
Abstract of a dissertation submitted by Diptish C. Chakravarty to the Graduate College, State University of Iowa, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacy.

TABLE II.—QUATERNARY SALTS OF 8-(DIALKYLAMINOALKOXY)-THEOBROMINES

R	n	R'	X	Yield, %	M. p.	Nitrogen, % Calcd.	Nitrogen, % Found
CH_3	2	CH_3	I	23	87– 88	17.1	17.1
		CH_3CH_2	I	20	126–128	16.5	16.5
		$C_6H_5CH_2$	Cl	12	131–133	17.5	17.5
CH_3CH_2	2	CH_3	I	20	106–107	16.0	16.0
		CH_3CH_2	I	25	96– 98	15.5	15.5
		$C_6H_5CH_2$	Cl	10	111–112	16.6	16.6
CH_3CH_2	3	CH_3	I	25	56– 57	15.5	15.4
		CH_3CH_2	I	24	76– 78	15.0	15.1
		$C_6H_5CH_2$	Cl	13	102–103	16.1	16.1

In the head-drop tests, groups of two rabbits were used. The concentrations of the solutions were adjusted to give a head-drop within a volume of 1–2 ml. The list of the compounds tested along with the results are included in Table III. The series of quaternary salts previously synthesised by these authors (4) were included in these tests.

DISCUSSION

The same procedure was satisfactory for preparing the methiodides, ethiodides, and benzchlorides of the dialkylaminoalkoxy derivatives of theobromine, which was not true for preparing the corresponding caffeine-substituted quaternaries (4). As indicated in Table III, the relative activities of the compounds differ in the mouse and the rabbit which may be ascribed to species variation, as previously reported (9). The theory that more than one quaternary ammonium group is essential for curare-like activity, as in (+)-tubocurarine, does not hold here. The distance-activity relationship theory, which has been criticized by later investigators, proposes that the distance between onium heads has a direct relationship to the attainment of maximum activity. This theory is also without support in this study, since the compounds have only one onium head. No conclusion is drawn as to why only one theobromine derivative exhibited activity while seven caffeine derivatives were active.

The degree of activity exhibited by the compounds did not warrant further pharmacological investigations.

TABLE III.—PARALYZING DOSES IN THE PRELIMINARY MOUSE SLOPING-SCREEN AND RABBIT HEAD-DROP TESTS

R	n	R'	X	Mouse Tests, Dose, 50 mg./Kg.	Rabbit Tests, mg./Kg.
		Theobromine Derivatives			
CH_3	2	CH_3	I	0[a]	0
		CH_3CH_2	I	0	0
		$C_6H_5CH_2$	Cl	0	0
CH_3CH_2	2	CH_3	I	0	0
		CH_3CH_2	I	0	0
		$C_6H_5CH_2$	Cl	0	0
CH_3CH_2	3	CH_3	I	72.5	17.4
		CH_3CH_2	I	0	0
		$C_6H_5CH_2$	Cl	0	0
		Caffeine Derivatives			
CH_3	2	CH_3	I	168.3	50.9
		CH_3CH_2	I	37.6	10.8
		$C_6H_5CH_2$	Cl	57 3	5.6
CH_3CH_2	2	CH_3	I	38 1	12.8
		CH_3CH_2	I	39.3	15 4
		$C_6H_5CH_2$	Cl	124 6	42.1
CH_3CH_2	3	CH_3	I	38 2	13 3
		CH_3CH_2	I	0	0
		$C_6H_5CH_2$	Cl	0	0
Flaxedil	..		..	4 1	0.47

[a] Inactive.

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Book Notices

Laboratory Guide in Pharmacology. By Harald G. O. Holck, Tom S. Miya, Norris W. Dunham, and George K. W. Yim. Burgess Publishing Co., 426 South 6th St., Minneapolis 15, Minn., 1959. iv + 115 pp. 22 x 27.5 cm. Paperbound. Price $3.50.

The manual is designed as an adjunct to lecture and textbook material, and only appropriate background information precedes the experimental procedures instead of detailed discussions of the pharmacological principles involved. Well-known procedures for preparing animals or isolated organs are not described in full detail. The manual is intended to acquaint the beginning student with various pharmacological principles in general without complicated and costly equipment. Most of the experiments are planned for student participation, and the procedures have been kept as simple as possible.

Pharmazeutisches Wörterbuch, Dritten Auflage. By Curt Hunnius. Walter De Gruyter & Co., Genthiner Strasse 13, Berlin W 35, Germany, 1959. xi + 731 pp. 12.5 x 18.5 cm. Price DM 32.

The first edition of this dictionary of pharmaceutical terms was published in 1950. The third edition has been enlarged, and includes definitions of a substantial number of terms not covered in the second edition, which was published in 1954. The definitions appear to be quite adequate, and for medicinal chemicals included in the *Deutschen Arzneibuch 6*, structural formulas, methods of preparation, and certain physical and chemical properties are given. Trademark names of drugs in this class are also included. The value of the dictionary is enhanced by an appendix including 17 tables of information of pharmaceutical interest. For example, antidotes, incompatibilities, maximum doses, and the relationship between different biological units are presented in tabular form.

Progress in Biochemistry. By Felix Haurowitz. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, N. Y., 1959. xii + 358 pp. 15 x 22.5 cm. Price $8.50.

This book is the fifth in a series of reports on the progress in biochemistry since 1914. In selecting material for this report, the author has given preference to the fundamental aspects of biochemistry rather than to those describing methods or applications.

Les Médicaments du Système Nerveux Cérébro-spinal. Edited by F. Mercier. Masson et Cie., 120, boulevard Saint-Germain, Paris 6^e, France, 1959. 573 pp. 16.5 x 24.5 cm. Paperbound. Price 5,800 fr.

This book (in French) covers the drugs affecting the central nervous system and includes discussions on related procedures such as electroencephalography and artificial hibernation. The text includes chapters on: general anesthesia, preanesthesia and sedation, hypnotics and neurosedatives, neuroleptics, analgesics, antiparkinsonism drugs, antiepileptic drugs, nervous stimulants, developed tolerance, toxicomania, neuropsychiatry, drugs acting on the neuromuscular junction, and local anesthetics. The book is intended to serve as a guide to students and practitioners. A good index (author and subject) is appended.

Précis de Thérapeutique et de Pharmacologie. 1959 Supplement to the 9th ed. (1950). By René Hazard. Masson et Cie., 120, boulevard Saint-Germain, Paris 6^e, France, 1959. 138 pp. 13.5 x 20 cm. Paperbound. Price 320 fr.

This supplement (in French) includes concise monographs on newer therapeutic agents. Generic, chemical, and trade names are given, but the manufacturers are not indicated.

An Introduction to Chemical Engineering. By Charles E. Littlejohn and George F. Meenaghan. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1959. xiii + 271 pp. Price $7.80.

This book is intended primarily as a beginning text for students in chemical engineering and related professions. It presents material with which the student should become familiar before undertaking a study of unit operations and thermodynamics. It attempts to explain some of the fundamentals upon which chemical engineering theory is based.

Source Book of Industrial Solvents. Vol. III Monohydric Alcohols. By Ibert Mellan. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1959. v + 276 pp. 15 x 23 cm. Price $10.

This book is the third in a series, and in the latest volume the physical properties, azeotropic mixtures, and uses of industrial alcohols are presented. Many significant tables and illustrations are included. Since alcohol solvents enter into nearly every kind of industrial activity, this book should enjoy wide acceptance for a convenient source of information on these substances.

Yardsticks for Industrial Research. By James B. Quinn. The Ronald Press Co., 15 East 26th St., New York 10, N. Y., 1959. v + 224 pp. 15 x 23 cm. Price $6.50.

The material presented in this book is based mainly upon a survey of practices of successful companies in evaluating industrial research output for management purposes. Using this approach, the author has developed a practical evaluation system designed to supply guidance to management. By means of the segmental approach, criteria and procedures are provided for both the technical and economic assessment of research output. The book probably will appeal most to research workers and executives who administer research programs.

Steroids. By LOUIS F. FIESER and MARY FIESER. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1959. xvii + 945 pp. 15 x 23.5 cm. Price $18.

A completely revised account of the entire field of steroids from the viewpoint of modern theory is presented in this book. It has been designed to replace "Natural Products Related to Phenanthrene," third edition (1949). The material in each chapter has been organized into a series of related topics unifying the discussion of the early history and the ensuing developments All literature has been covered to about February 1959. Steroids are of especial interest in the pharmaceutical field because so many medicinal chemicals fall within this classification. The book has been designed in a very competent manner, which will appeal both to experts and students whose main interests lie in the field of steroid chemistry.

Elementary Statistics With Applications in Medicine and the Biological Sciences. By FREDERICK E. CROXTON. Dover Publications, Inc., 180 Varick St., New York 14, N. Y., 1959. vii + 376 pp. 13 5 x 20 5 cm. Paperbound. Price $1.95.

Assuming only a modest knowledge of mathematics and no prior knowledge of statistics, this book is designed to show how statisticians arrive at their results, how to interpret statistics, and how to test their validity. Numerous examples are given from fields of investigation that are familiar to most research workers in the biological sciences.

Translation from German for Chemists. By H. H. NEVILLE and W. E. YUILL. Interscience Publishers, Inc , 250 Fifth Ave., New York 1, N. Y., 1959. xi + 139 pp. 13.5 x 18 cm. Price $2.50.

The aim of this book is not to teach the chemist German, but to equip him with a technique for reading, translating, and understanding German chemical material. Only those features of the language which are necessary for an intelligent use of the dictionary are presented. No previous knowledge of German is required to use this book, but some background would make the subject matter easier to follow.

Brief Course in Organic Chemistry. 2nd ed. By LYELL C. BEHR, REYNOLD C. FUSON, and HAROLD R. SNYDER. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N Y., 1959. viii + 289 pp. 15 x 23 cm. Price $5.75.

This revised and expanded edition of the text presents an introduction to organic chemistry and includes the application of organic compounds. The revision contains references to many substances of practical value such as vitamins, hormones, other natural products, medicinals, agricultural chemicals, and other substances of biological interest. New chapters on homologous series, naturally occurring esters, and sulfur compounds have been added, and there are new illustrative photographs and drawings of molecular structures. Aromatic and heterocyclic compounds are introduced early and the similarities among aliphatic, aromatic, and heterocyclic compounds, rather than their differences, are stressed.

Textbook of Toxicology. By KENNETH P. DU BOIS. Oxford University Press, 417 Fifth Ave., New York 16, N. Y., 1959. x + 302 pp. 14 x 21.5 cm. Price $6.50.

This book was written to fill the need for a textbook on toxicology containing subject matter in a concise, understandable form. "*Textbook of Toxicology*" provides a concise but thorough coverage of factual information concerning the major classes of the mode of action of poisons discussed at the cellular level. The practical circumstances under which poisoning can occur, diagnosis of poison, and treatment are also adequately discussed. The text stresses the toxic effects of the classes of chemical compounds rather than attempting a complete coverage of all toxic chemical agents. Chapters on newer subjects such as ionizing radiations, household poisons, and pesticides are included as well as subjects of long-standing importance such as metals, air-borne poisons, solvents, and plant and animal poisons. The medicolegal aspects, history, and general principles of toxicology are also discussed. The book is designed for use by medical students, graduate students, and advanced undergraduates, but it will also be of great value as a quick reference work in emergencies.

Anatomy and Physiology. Vol. I. By EDWIN B. STEEN and ASHLEY MONTAGU. Barnes & Noble, 105 Fifth Ave., New York 3, N. Y., 1959. xv + 332 pp. 13.5 x 21 cm. Paperbound. Price $2.50.

This book, one of the College Outline Series, presents a survey covering cells, tissues, integument, skeletal, muscular and digestive systems, blood, lymph, and circulatory system. It is keyed to standard textbooks on anatomy and physiology.

Carcinogenesis. Mechanisms of Action. Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. xii + 336 pp. 13.5 x 20.5 cm. Price $9.50.

This book includes a series of papers presented during the 1958 Ciba Foundation Symposium which was held in London. All of the papers presented were prepared by experts in the field of carcinogenesis.

Regulation of Cell Metabolism. Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. xi + 387 pp. 13.5 x 20.5 cm. Price $9.50.

A series of papers presented to a 1958 symposium on the regulation of cell metabolism is included in this book. This new symposium from the Ciba Foundation draws upon the collective experience of 39 internationally known authorities to present the latest research findings in the regulation of cell metabolism. The book is profusely illustrated with halftones and charts which clarify and augment the textual discussions. This volume should be of special interest to everyone involved in basic cell research.

Scientific Edition

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A Simplified Procedure for the Determination of Vitamin A*

By JOSEPH A. NAPOLI, BERNARD Z. SENKOWSKI, *and* ALEX. E. MOTCHANE

A modification of the U. S. P. XV procedure is described. The proposed procedure shortens considerably the time for analysis. It also improves the precision of the unsaponifiable values by at least a factor of two. The precision of the Morton-Stubbs correction factor remains unchanged. The method is shown to be applicable to a wide range of products. A discussion of solvent purification and instrument checking and calibration is included.

THE WIDELY USED PROCEDURE for the quantitative analysis of vitamin A in various products is described in the U. S P XV (1). It represents the experience accumulated in numerous industrial and scientific laboratories over the past ten or fifteen years The improvement proposed consists of the use of a single extraction in place of the multiple extractions of the U S P XV procedure As a consequence, a notable increase in the precision is obtained The U S P XV reports an expected discrepancy in results of different laboratories using the prescribed method of approximately 2 S D = ±8 per cent at $P = 0.05$ With this magnitude of error, it has been necessary to carry out four determinations in order to obtain a corrected average value of about ±2 per cent ($P = 0.05$).

Several attempts have been made toward improving the method and its precision (2–4) but none has found its way into general practice In particular, Mulder's (5) recent work should be mentioned for a thorough analysis of the problem and a novel solution. Our work was done very nearly at the same time.

* Received July 16, 1959, from the Control Spectrophotometric and Physical Chemistry Laboratories of Hoffmann La Roche Inc., Nutley N J

Our sincere thanks to L G L Shafer, Director of Products Control for his helpful advice and to Dr F Forrester of the Technical Development Department, Analytical Division for supplying the data in Table III

The modification of the U. S P. XV method which we have developed eliminates at least one major source of variability and significantly cuts the time for the analysis At the same time the precision of the unsaponified values is at least doubled.

RESULTS

Several series of comparisons are presented between results obtained by the new method and by the U S P XV

The variations of the individual potency values in Table I are obviously completely out of range from each other and could be treated together only on a relative scale Such a uniform scale is easily obtained by adjusting all values to the same arbitrarily chosen level, i e, using appropriate factors to make the averages of individual samples to read, for instance, 1 000 or any other convenient figure. Conventional simple statistics then become meaningful, keeping in mind that although the variations between samples have been suppressed, the number of different groups of samples used reduces the number of the degrees of freedom available for comparison.

This procedure is justified by the technique of measurement, which is based on spectrophotometric readings, and dictated mainly by its limitations. All such readings are in the range of E between 0 5 and 0 8, and are, in practice, even within somewhat closer limits (see footnote, Table V) This is accomplished, as is well known, by adjusting weights and dilutions The direct readings could not be compared at all without adjustment for weights. The error is related directly to the experimental results which are these very closely similar readings The differences between the samples are given, on the other hand, mainly by the weights and dilution factors by which all errors are being multiplied at the same time as the readings The actual variations in the final results must be, therefore, proportional to the dilution factors, i e, in the end, to the amount of vitamin A They are obviously very different with different samples, whereas the relati-

errors remain the same. These differences in factors are seen to be accidental, and consequently irrelevant, as far as the relative error is concerned.

The precision indexes were obtained by the analysis of variance. The results are collected in Fig. 1 and Table V.

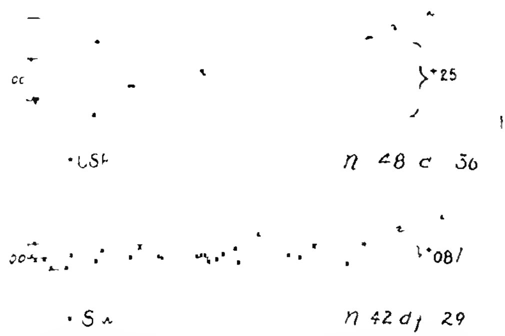

Fig. 1.—Random errors in vitamin A determinations, multiple extraction (U. S. P. XV) *vs.* single (S. W. E.)

DISCUSSION

In the discussion of our results, we wish to stress two points. The first point is purely technical: a single extraction obviously eliminates one source of error, namely multiple transfers of ether extracts. Such transfers can be made quantitatively, but this may be difficult to achieve with uniform precision in routine operations. This source of error has never been properly evaluated. The number of shakings to which at least some of the vitamin A present is subjected is reduced considerably, and this also may be of importance.

The second point refers to the system of solvents used. The extraction with one large volume of ether (150 ml.) radically changes the composition of the phases. Vitamin A is actually extracted in one operation, the phases being pure water—containing soap, if present—and ether containing 20% alcohol plus, possibly, very dilute soap. There is no question that this extraction is quantitative, i. e., that the distribution is decisively shifted in favor of the ether layer.

It is not known at present which of these changes is of greater importance. The second is more fundamental, but not necessarily more decisive.

The maximum absorption, as shown in Table IV, was found as high in the new procedure (S. W. E.[1]) as in the U. S. P. XV. The regression coefficient of S. W. E. on U. S. P. XV values was, for the data in Table I, 1.0005 ± 0.002, Table II, 1.006 ± 0.014, the limits of error being 2 S. D., as in Table V. We are investigating the possibility for which there are a few indications that a slightly higher recovery might be possible. The important point, for the time being, remains that the recovery by the new procedure is at least as good as by U. S. P. XV.

The precision of the uncorrected values, $E(1\%, 1\ cm.)$, is, on the other hand, considerably better than the U. S. P. XV, as seen from Table V. The error was about two to three times smaller and this result highly significant (at the level of $P = 2.5$ to 0.5%), (Fig. 1).

[1] S. W. E. is used to denote the single water ether method as proposed in this report.

On the other hand, the precision of the correction factor ($F.\ MS = Ac\ corr./A\ unc.$) was not improved (see Tables). The number of data seems to be sufficient to consider this difference as real. At present, we have no definite explanation for this. It appears that we have obtained a separation of the effect on the reading at the maximum, and of the effect on readings on the slopes of the curve: the former became considerably less variable, whereas the latter, or at least their combined effect, remained as variable as before. We abstain from discussing several possibilities which, for the time being, must be speculative only. As a practical conclusion, the precision of the analytical results will be improved with respect to U. S. P. XV when the correction factor is greater than 0.97, since then the correction is omitted: in short, when dealing with material of high spectral purity. The studies now in progress may show the way to improve precision in all cases.

The last point we wish to mention, ratio S/W, represents an additional proof of the soundness of the new procedure. The height of the maximum of the whole sample (W) before, and the height (S) after saponification are important physical data which are used much too little. Provided the absorption measured are due to vitamin A only, the absorption after saponification must be higher than before, since the molecular absorption of the vitamin A alcohol is higher than of the esters. The following comparison (Table VI) is based on data from Table I.

The independent significance of this index is in the possibility afforded for checking the analysis when the whole value is available, as is often the case, without reference to another sample or standard. The measurement of a direct dilution of the whole sample is generally easy to make with relatively high precision and accuracy. A ratio near 1.04 confirms the validity of the reading after saponification, and conversely, and perhaps, more critically, a significantly lower ratio is a sure indication of an error, loss, or a discrepancy of some kind. A value slightly higher than 1.04 is the ratio of the molecular absorption of the free vitamin A alcohol and of the acetate, which can be derived for instance, from data published by Cama, *et al.* (6), as early as 1951. In our experience it holds for the palmitate as well as the acetate, and it was found helpful in our laboratories, as indicated, at least since that time.

CONCLUSIONS

Replacement of the four ether extractions called for in the U.S.P. XV procedure for vitamin A determination by one extraction with a fivefold volume of ether results in a partial, but significant, improvement in operation and results. Notably, two things are achieved: the time of analysis is reduced to almost one half, and the precision of the absorption measured is increased two to three times. The full benefit of these improvements is felt in cases where the measured values represent the final result, i. e., with high purity vitamin A, diluted or undiluted, showing a U. S. P. purity factor $F.\ MS$ of more than 0.97, and therefore not requiring correction (1).

On the other hand the precision of the correction factor was not improved. In this way, apparently,

a separation has appeared for the first time in experimental data of variations affecting the maximum of the absorption curve, as distinct from the slopes This matter shall be discussed later on the basis of new experiments Considerable improvement seems possible in this direction

PROCEDURE

Sampling.—A sample of not less than 100 mg is recommended in order to have a precision of 0 1% or better depending on the balance used Also, the weighed sample should contain not less than 10,000 units of vitamin A

High potency material, crystalline or liquid, as such or diluted in oil, is best weighed directly into a volumetric flask (25-ml), dissolved by adding 1 ml of ethyl ether, and bringing to mark with isopropyl alcohol A 5-ml aliquot is transferred to the saponification flask Powders are sampled in a weighing bottle, transferred to a saponification flask, and 7 ml glycerin added Multivitamin liquid preparations are weighed directly into the saponification flask In preparations containing vitamin

TABLE I —U S P XV *vs* SINGLE EXTRACTION PROCEDURE (S W E)

Sample (u /Gm) × 10⁶	E (1%, 1 cm) U S P XV Unsaponifiable Fraction	*f* (MS)[a]	*S/W*[b]	E (1%, 1 cm) S W E Unsaponifiable Fraction	*f* (MS)[a]	*S/W*[b]	Direct Whole u /Gm
2 9	1571	1 0148		1559	0 9739		1495
	1571	1 0150		1558	0 9575		
	Av 1571	1 0149	1 050	1561	0 9827		
				1558	0 9705		
				Av 1559	0 9712	1 043	
1 8	931 5	0 9630		936 9	0 9998		906 5
	919 2	0 9602		945 6	1 0066		
Sample 1	931 8	0 9596		945 6	1 0113		
	923 9	0 9459		946 8	1 0045		
	Av. 926 9	0 9572	1 022	Av 943 7	1 0056	1 041	
1 8	964 1	1 0000		960 0	0 9909		919 0
	958 2	0 9950		966 0	0 9950		917 0
Sample 2	982 3	0 9527		Av 963 0	0 9930	1 049	Av 918 0
	940 7	1 0263					
	Av. 961 4	0 9950	1 047				
1 0	618 1	0 9324		625 7	0 9630		602 1
Sample 1	618 7	0 9275		625 7	0 9581		
	609 0	0 9350		626 3	0 9650		
	609 3	0 9343		630 0	0 9689		
	Av. 613 8	0 9301	1 019	Av 626 9	0 9640	1 041	
1 0	615 4	0 9221		620 9	0 9643		585 8
	618 9	0 9534		619 9	0 9725		589 1
Sample 2	612 8	0 9677		Av 620 4	0 9684	1 056	Av 587 5
	612 8	0 9625					
	Av 615 0	0 9464	1 067				
0 5	323 8	0 9916		323 4	0 9905		
(Dry pellets)	325 3	1 0038		323 4	0 9940		
	326 3	0 9739		321 7	1 0048		
	324 3	0 9718		323 6	1 0069		
	Av. 324 9	0 9853		Av 323 0	0 9992		
				320 0	0 9820		
				320 8	1 0011		
				Av. 320 4	0 9916		
0 325	202 6	0 9793		197 5	0 9984		
(Dry pellets)	193 4	0 9582		195 9	1 0167		
Sample 1	202 8	0 9643		Av 196 7	1 0076		
	202 7	0 9718					
	Av 200 4	0 9684					
0 325	216 5	1 0038		208 2	0 9670		
(Dry pellets)	210 4	0 9909		208 9	0 9677		
Sample 2	214 1	0 9691		208 1	0 9848		
	197 4	0 9827		211 8	0 9452		
	Av 209 6	0 9866		Av 209 3	0 9662		
0 10	53 03	1 011		54 01	1 067		51 99
U S P Vitamin A	51 85	1 047		53 85	1 059		51 07
Reference	53 64	1 054		53 94	1 058		50 75
Soln (1)	52 75	1 050		53 92	1 059		51 85
	51 22	1 013		Av 53 93	1 056	1 053	50 85
	53 52	1 058					50 66
	53 72	1 067					51 95
	52 10	1 053					51 10
	53 55	1 055					50 88
	Av 52 82	1.045	1 031				Av 51 23

TABLE I. (*Continued*)

Sample (μ Gm) × 10⁵	E (1%, 1 cm) U S P XV Unsaponifiable Fraction	f (MS)[a]	S/W[b]	E (1%, 1 cm) S W. E Unsaponifiable Fraction	F (MS)[a]	S/W[b]	Direct Whole μ/Gm
0.04	33.71	0.7462		33.73	0.7830		
Vitamin A	33.87	0.7599		33.41	0.7783	.	
"Natural"	Av. 33.79	0.7531	1.027	Av. 33.57	0.7807	1.020	32.9
from fish oils							
0.001	5.933	0.9098		5.827	0.9166	.	
Vitamin A in	5.827	0.8873		5.818	0.9132		
aqueous	5.828	0.9398		5.789	0.8996	.	
multi-	Av. 5.863	0.9118		5.769	0.9187		
vitamin		.		Av. 5.801	0.9120	..	...
prepn							
0.06	37.51	0.9282		38.70	0.9105	..	.
Vitamin A	37.50	0.8996		39.22	0.8982	.	.
with	38.25	0.9207		Av. 38.95	0.9044	.	.
β-carotene	38.38	0.9125				.	
in oil	Av. 37.91	0.9153					..

[a] A corr/A uncorr (1) $F\text{(MS)} = A_{325\,corr} = \frac{6.815\,(A_{325}) - 2.5554\,(A_{310}) - 2.60\,(A_{334})}{A_{325}}$

[b] $S/W = \frac{A_{325}\text{ (after saponification)}}{A_{325}\text{ (direct measurement, no saponification)}}$, *i e* unsap fraction over direct whole

Data in this table refer to samples of widely different potencies (18 values from 12 samples as analyzed by two chemists in Laboratories I and II)

A and β-carotene in oil, the carotene is removed by weighing *about* 1 Gm[1] *into* an amber, glass-stoppered centrifuge tube, adding 20 ml. of isopropyl alcohol, gassing with nitrogen, shaking on a shaking machine for ten minutes, and centrifuging A 5-ml aliquot of the clear supernatant solvent is placed in the saponification flask

Saponification.—To the sample in the 125-ml saponification flask is added sufficient 95% U S P alcohol to make a total volume of about 30 ml, followed by 3 ml of 50% w/w potassium hydroxide

Displace the air by nitrogen, reflux on a steam bath for thirty minutes, and cool rapidly under the tap to room temperature

Extraction.—With all samples except dry vitamin A powders, add 30 ml of water into the saponification flask and transfer quantitatively into an amber 250-ml separatory funnel (Squibb type[2])

In the case of dry vitamin A powders, add only 20 ml water before transferring, then rinse the saponification flask with the remaining 10 ml of water in order to pick up any deposit which may have formed during saponification Now add 2 Gm hydrated sodium sulfate, finely powdered

Rinse the saponification flask with three 50-ml portions of ethyl ether, collecting them in the separator (Rinse the top of the flask while it is inverted over the separatory funnel to prevent run back)

Displace air with nitrogen, stopper, shake vigorously for about two minutes, and let stand till the layers separate completely, five minutes usually suffices Add a few drops of water if the boundary is not clearly visible

Washing.—Discard the aqueous layer leaving one drop above the stopcock Add 60 ml distilled water, swirl gently, and wait till the layers separate completely Discard the water, add another 50 ml of water, displace the air with nitrogen, close, and gently tumble the funnel Repeat this treatment two more times, shaking a little more vigorously Discard the water layers

Dilution.—Transfer the entire ethyl ether extract to a 250-ml amber volumetric flask, rinsing the separator several times with small portions of ethyl ether and collecting them in the flask, and bring to mark with ether Subdilute with isopropyl alcohol The final concentration of ether in the measured solution should not exceed 5% This solution should contain between 10 and 15 units of vitamin per ml

Measurement.—Measure the absorbance at the following wavelengths four times each, using a suitable spectrophotometer. 310 mμ, 325 mμ, 334 mμ Average the values for each wavelength and proceed with the calculation

Calculate using the following expression:

$$E(1\%,\ 1\text{ cm}),\ (325\text{ m}\mu) = \frac{E \times V}{W \times 100} \times \frac{1}{L}$$

where E = average absorbance at 325 mμ, V = calculated final volume containing W (grams) of sample, in ml, W = weight of sample in grams, and L = path length in cm

Correction for irrelevent absorption calculate according to U S P XV (1)

$$A_{(325\ corr)} = 6.815\,(A_{325}) - 2.555\,(A_{310}) - 1.260\,(A_{334})$$

In the case of oils containing β carotene the volume of isopropyl alcohol added to dissolve the vitamin A and separate it from the β carotene is increased by the oil present This volume change must be corrected for as follows: $V_c = 20 + \Delta V$, ΔV = weight of sample in grams × 1.16, V_c = volume corrected, 1.16 = the increase of the volume of 20 ml of isopropyl alcohol per 1 Gm of oil containing β carotene and vitamin A as obtained from the experimentally determined straight line which passed through the origin.

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[1] This weight should not exceed 1.2 Gm

[2] No grease is employed the stopcocks (1 mm bore) are lubricated with water only.

TABLE II.—U. S. P. XV *vs.* S. W. E. DRY VITAMIN A PELLETS[a]

Sample	U. S. P. XV E (1%, 1 cm.)	U. S. P. XV F (MS)[b]	S. W. E. E (1%, 1 cm.)	S. W. E. F (MS)[b]
1	204 7	0 9849	204 8	0 9688
	206 0	1 0045	210 4	0 9982
	Av 205 4	0 9947	207 6	0 9835
2	200 8	0 9415	199 3	0 9408
	199 2	0 9135	200 1	0 9492
	Av 200 0	0 9275	199 7	0 9450
3	195 6	1 0031	199 1	0 9877
	192 8	1 0003	199 6	0 9800
	Av 194 2	1 0017	199 4	0 9839
4	205 6	1 0129	204 5	0 9828
	203 3	1 0171	205 4	0 9786
	Av 204 5	1 0150	205 0	0 9807
5	216 1	0 9702	214 6	0 9541
	212 9	0 9847	210 0	0 9611
	Av. 214 9	0 9772	212 3	0 9576
6	215 7	0 9415	214 9	0 9205
	215 0	0 9534	216 2	0 9492
	Av 215 4	0 9475	215 6	0 9349
7	213 7	0 9902	215 7	0 9807
	216 1	0 9882	205 4	1 0213
	Av 214 9	0 9892	210 6	1 0010
8	210 2	1 0003	210 6	0 9723
	206 8	0 9660	212 6	0 9807
	Av. 208 5	0 9832	211 6	0 9765
9	216 5	1 0038	213 1	0 9691
	214 1	0 9909	199 3	0 9827
	Av 215 3	0 9974	206 2	0 9759
10	202 7	0 9575	213 4	0 9664
	208 2	0 9357	212 4	0 9030
	Av. 205 5	0 9466	212 9	0 9347
11	209 9	0 9486	213 3	0 9309
	209 6	0 9337	215 8	0 9323
	Av 209 8	0 9412	214 6	0 9316
12	206 3	0 9364	209 7	0 9521
	203 0	0 9228	210 7	0 9166
	Av. 204 7	0 9296	210.2	0 9344
13	210 5	0 9159	212 1	0 9589
	212 8	0 9228	212 7	0 9377
	Av. 211 7	0 9194	212 4	0 9483
14	215 4	0 9596	222 9	0 9486
	211 6	0 9534	223 7	0 9228
	Av. 213 5	0 9517	223 3	0 9357
15	211 2	0 9950	215 9	0 9670
	211 1	0 9534	204 8	0 9589
	Av. 211 2	0 9742	210 4	0 9630
16	209 2	0.9493	209 9	0 9875
	210 5	0 9561	203 5	1 0148
	Av. 209 9	0 9527	206 7	1 0012
17	211 2	1 0058	202 9	0 9889
	212 8	1 0079	205 7	0 9834
	Av 212 0	1.0069	204.3	0 9862
18	205 7	0 9282	215 4	1 0040
	207 1	0.9405	215 7	0 9507
	Av. 206 4	0 9344	215 6	0 9774
19	218 1	0 9759	221 1	0 9759
	201 3	0 9317	223 7	0 9602
	Av. 209 7	0 9538	222 4	0 9680
20	222 6	0 9923	222 8	1 0180
	222 0	1 0093	211 2	0 9609
	Av. 222 3	1.0008	217 0	0 9894
21	210 4	0 9820	223 8	0 9596
	211 0	1 0209	224 4	0 9848
	Av 210 7	1 0015	224 1	0 9722
22	208 2	1 0395	199 2	0 9912
	208 7	1 0269	200 6	1 0206
	Av 208 5	1 0332	199 9	1 0059

[a] Duplicate measurements on 22 lots of vitamin A pellets as analyzed in Laboratory No I [b] See footnote[a] Table I

EXPERIMENTAL

Apparatus.—Of the Beckman DU spectrophotometers used, one was equipped with a photomultiplier detector and another was equipped with a conventional photocell In order to obtain greater source stability, our hydrogen lamp power supply units are fed from a 1 KVA Sola constant voltage transformer

The importance of wavelength calibration cannot be overemphasized and particular care is taken in checking the calibration of our instruments and adjusting the scale. A mercury discharge lamp (supplied by the instrument manufacturer) is used to check periodically our wavelength settings.

The following is a typical control run

Known Mercury Lines mμ	Mercury Lines, Found D265[a]	506	40735	113907[a]
253 65	253 79	253 70	253 74	253 68
289 36	289 49	289 50	289 45	289 25
312 57	312 67	312 62	312 77	312 54
313 15	313 33	313 29	313 23	313 15
334 15	334 05	334 13	334 13	334 14

[a] Instruments used in this study

Any notable deviation from the expected wavelength is corrected for by following the directions supplied by the manufacturer

TABLE III.—COMPARISON OF RESULTS BY SINGLE EXTRACTION (S W E.) AND U. S P. XV METHOD

High purity Vitamin A $\left(0.97 \leq \frac{A \text{ corr.}}{A} \leq 1.03\right)$

$$\frac{\text{S W E}}{\text{U S P XV}} \text{ (average of 141 samples)} = 1.001$$

Standard Deviation = ±4%, the magnitude of which is determined by the larger error of the U. S P method.

Instrument Settings.—Inaccurate instrument settings can be disastrous in this analysis and special care must be exercised in setting the wavelengths accurately. The slit settings are of great importance and care must be taken with this setting, too. It is for that reason, as well as slight source instability, that it is recommended that each reading be taken four times and the average of the four readings used in the subsequent calculations Any value which deviates more than 2% from the other values is omitted and further checks are made to substantiate the value.

A typical slit program is as follows: 310 mμ, 0 22 mm.; 325 mμ, 0 20 mm ; 334 mμ, 0 18 mm.

Calibration of Density Readings.—A solution containing 20 220 mg of Bureau of Standards potassium dichromate, dried at 140° to constant weight, in 1,000 ml of 0 05 N potassium hydroxide was prepared a

TABLE IV —SUMMARY OF THE COMPARISONS OF THE SINGLE EXTRACTION PROCEDURE (S. W. E.) AND THE U. S P. XV

Table No	Potency Range 10^3 u /Gm.	E (1%, 1 cm) Ratio S W E /U S P. XV[a]
I Liquid preparations, 46 values	0 01 to 2 9	(9)[b] 1 005 (12) 1 000
II Vitamin A pellets, 44 values	0 38	(22) 1 007
III High potency vitamin A, 141 values	1 3 to 2 9	(141) 1 000

[a] Figures in parentheses are the number of samples
[b] Liquid preparations

TABLE V —PRECISION (2 S D) OF U S P XV AND OF S W E. (SINGLE EXTRACTION) REFERRED TO SAMPLE AVERAGE = 100

Samples	Degrees of Freedom	Precision of	2 S D % U S P XV	S W E	Variance Ratio	F Level
12 (Tab 1[a])	36	E(1%, 1 cm)	2 5	0 8	10 6	2 6 (0 005)
		F (MS)	3 0	2 5	1 2	
22 (Tab 2)	22	E(1%, 1 cm)	5 7	3 7	2 4	2 36 (0 025)
		F(MS)	3 0	4 0	0 75	

[a] Sample	1	2	3	4	5	6	7	8	9	10	11	12
E(1%, 1 cm)	1 560	945	960	625	625	325	200	200	54	33	555	39
Readings (E 1 cm), approx												
U S P	0 65	0 70	0 60/0 76	0 60/0 67	0 56/0 70	0 69	0 62/0 79	0 70	0 53	0 65	0 69	0 57/0 74
S W E	0 65	0 67	0 50/0 55	0 56	0 58	0 68	0 54	0 52/0 67	0 71	0 70	0 68	0 78

F(MS), Morton Stubbs correction factor, based on U S P XV (1) calculated directly from ratios = A (corr)/A (325)

TABLE VI —RATIO S/W, UNCORRECTED (UNSAP. TO WHOLE)

Sample	1	2	3	4	5	9	10	Av
U S P XV	1 050	1 022	1 047	1 019	1 047	1 031	1 027	1 030
S W. E.	1 043	1 041	1 049	1.041	1 056	1 053	1 020	1 014

The difference between the averages (S D = 0 0124) is not significant at P = 0 05. The ratio is in both cases about 1.04.

measured at 372 5–373 mμ *vs* 0 05 N potassium hydroxide

Instrument	E (1%/1 cm)
D265	249 4
506	249 3
40735	249 4
113907	249 3

These solutions were sealed in 5-cc ampuls and monthly checks are performed on all instruments using these solutions A graphical record is kept and any abnormal deviations are immediately investigated The values shown above agree quite well with the values published by Cama, Collins, and Morton (2) Morton's Beckman, A = 248 2, average of all instruments (in Morton's paper) A = 247 4

Stability of the instruments is also enhanced by the use of large capacity, slow discharge rate, six-volt batteries

Quartz Cells.—(Fused Quartz cells are recommended) The quartz cells employed were matched for absorbance by comparing them, after suitable cleaning, using the same solvent in each

Cleaning is performed by thorough rinsing with the solvent used (isopropanol in the case of water-immiscible solvents) followed in succession by distilled water, soaking in a mild detergent for about one-half hour and adequate distilled water rinsing The cells are then checked, one *versus* the other. If detergent cleaning does not suffice, immerse them in 30% fuming sulfuric acid for about twenty minutes[4] This is followed by thorough rinsing with distilled water and checking

The path length of the cells should be known to at least ±0 5% The stated value of the cells was checked by two independent methods

First a cell was measured using standard chromate at the maximum (373 mμ) This value was then confirmed with greater precision by half-filling the cell with mercury and measuring, using a microscope micrometer, the distance between the mercury-quartz boundaries at opposite faces

Solvents.—(*a*) The ethyl alcohol used is 95% U S P ethanol, aldehyde-free (*b*) The ethyl ether used is Mallinckrodt (black label) special peroxide-free grade (AR) analytical reagent Daily checks for peroxides are performed with KI solution, however, a more sensitive check is to keep a solution of vitamin A in the ether overnight, or longer, in the refrigerator This solution should be stable for several days in pure peroxide-free ethyl ether. (*c*) Shell isopropyl alcohol, or one of comparable spectral purity, is passed through a Norite column containing five pounds of Norite for five gallons of isopropanol at the rate of one gallon per day. This

[4] Fused cells only

is followed by a simple distillation through a 1-meter glass helix column at the rate of one gallon per day. This treatment produces a solvent of adequate spectral purity.

Glassware.—All glassware used, except for the pipets, is amber, light-resistant. Careful cleaning is essential. The cleaning procedure recommended is as follows: Rinsing with tap water; soaking in a Calgonite bath overnight; rinsing with tap water; shaking each piece with concentrated chromic acid solution; and rinsing with tap water, followed by distilled water until all the acid is rinsed out. (At least 5 rinsings of each.)

Balance.—Periodic checks on the balance and weights are performed by using standardized Bureau of Standards weights.

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Ethylene Oxide Sterilization of Spores in Hygroscopic Environments*

By JOHN B. OPFELL, JOHN P. HOHMANN, and ARTHUR B. LATHAM

Bacillus globigii spores were exposed to ethylene oxide after being dried on several types of surfaces. The viability tests, after exposure, showed significant effects were produced by the presence of the hygroscopic substances, glycerin, and filter paper. Absence of hygroscopic substances appeared to increase the resistance of *B. globigii* spores to gaseous ethylene oxide sterilization.

WATER VAPOR is a factor in ethylene oxide sterilization. Some writers (1, 10, 20) contend that moisture improves the effectiveness whereas others (6–9, 12, 13) have observed the contrary. Methods (5, 11, 14, 20) of inoculating objects to be sterilized have differed among the several investigations. The effect of the inoculation method on the evaluation of the sterilization process was studied. The results of this study suggest a possible explanation for the contradictory observations.

For more than twenty years ethylene oxide has been used to sterilize temperature-sensitive medical equipment. Several investigations of processing conditions have been made. Grundy and co-workers (11) found ethylene oxide sterilized inoculums of 20,000 spores on filter paper in two hours at 26.7°. The concentration of ethylene oxide used was 1,250 mg. per liter. The immediate environment of the spore contained lactose as well as filter paper. Lloyd and Thompson (14) reported that two hours at 55° in 1,000 mg. per liter of ethylene oxide were sufficient to sterilize 10^6 *B. globigii*, 5×10^5 *Cl. sporogenes*, and 10^5 *B. stearothermophilus* spores on filter paper strips when the relative humidity of the ethylene oxide was between 30 and 50 per cent. Eisman (5) showed that ethylene oxide does not completely penetrate large masses of clothing. This is perhaps not too surprising when one considers the mass of absorbed water in clothing which has not been desiccated. At 30°, water as a liquid can absorb as much as 0.4 Gm. (15) of ethylene oxide per Gm. of water. Hall (6–9) claims that spices are easier to sterilize when they are dry. The extent of drying necessary was not specified. The experiment to be described was motivated by observing that washed *B. globigii* spores in large inoculums desiccated on polystyrene could not be inactivated by a process which sterilized these spores dried on filter paper.

The standards for evaluating sterilization are described in the current edition of the "United States Pharmacopeia" (19). Meaningful sterility tests must conform to these standards. In addition, sterility tests are meaningful only when the test organism is specified and is used under defined conditions. As the test organism, most investigations (5, 11, 14, 17, 20) have used spores of *Bacillus subtilis* var. *niger* strain *B. globigii* (ATCC 9372) (3). This organism was used in the experiment to be described. This spore is not necessarily the one most resistant (5) to ethylene oxide exposure under all conditions.

The number of cells in the inoculum and the method of applying the inoculum to the test object are important factors in evaluating a sterilization process. The appropriate number of cells which should be used depends on the number which might occur naturally on the object. The resistance of an inoculum measured by the period of exposure required to render the object sterile,

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depends on the size of the inoculum. For example, assume that the probability that a spore selected at random from the inoculum would survive an exposure of t minutes under specified processing conditions is:

$$p = e^{-kt} \qquad \text{(Eq. 1)}$$

(The constant k has units of reciprocal minutes.) One can show that the probability of failing to sterilize the object after an exposure of t minutes under the same processing conditions is approximately

$$P = Ne^{-kt} \qquad \text{(Eq. 2)}$$

for large exposure times. The number of spores in the inoculum is N. To reduce the probability of failing to sterilize to a very low level, either the size of the inoculum must be reduced or the exposure time must be sufficiently increased. Equations 1 and 2 have been amply confirmed (2, 4, 20) for both ethylene oxide and steam sterilization. In practice, N cannot be reduced below the maximum microorganism population which might naturally inhabit the object. Because microorganisms have finite volumes, N cannot be infinitely large. Usually N is chosen (11, 14) sufficiently large to include an ample safety factor over the natural population size observed to exist in representative samples of similar objects.

Some experimenters (5, 20) have used suspensions of spores deposited and dried on the surface to be sterilized. Others have used small pieces of paper (11) or fabric (20) which have been inoculated with spore suspensions and dried before insertion into containers to be sterilized. Still others have dried suspensions of spores in starch paste or physiological saline, lactose, or glycerine solution on the surface to be inoculated.

Variations in method of inoculating surfaces to be used in proving sterilization have been significant. The effect of such variations was measured in the experiment to be described. To the extent possible, the size of the inoculum was uniform and the ethylene oxide concentration was varied in a systematic manner.

METHODS

The inside surfaces of tubes one-fourth inch in internal diameter and seven-eighths inch in length were inoculated with *B. globigii* spores. Except for category B, described in Table I, the spores were dried from suspension in distilled water. The spores were washed in distilled water before the final suspension was prepared to free them of hygroscopic and other substances which are not part of their innate structure. In category B the suspension contained 2% glycerin U. S. P. In category C the spores were deposited on the filter paper only. The tubes were inoculated with uniform volumes of the stock spore suspension. The number of viable spores recovered by a single rinse was determined by a plate-counting technique using a representative inoculated tube after it had dried for twenty-four hours in a 32° incubator. While the recovery of spores in categories B, C, and D was roughly the same, relatively fewer were recovered when dried on polystyrene from suspension in distilled water.

For each concentration of ethylene oxide shown in Table I the indicated number of tubes representing each inoculation method were placed together in a 200-ml. glass flask immersed in an air bath thermostat. After evacuation with an oil-sealed mechanical pump, the indicated amount of dry pure ethylene oxide vapor was admitted to the flask. The concentration was computed from the measured pressure in the flask at 37°, the temperature of the air bath after the system had reached equilibrium Equilibrium was attained in a matter of a very few minutes. The ethylene oxide was left in contact with the inoculated tubes for seventeen hours at 37 ± 1° before it was removed by evacuation and replaced with air filtered through a sterile cotton filter.

Each of the treated tubes was tested for sterility using procedures described in the "United States Pharmacopeia" (19). Each was placed in a test tube containing 40 ml. of fluid thioglycollate medium and incubated there for seven days at 32°. In every experiment two untreated but inoculated controls from category D were combined with two polystyrene tubes, which had not been inoculated, in ten ml. of the medium. The two polystyrene tubes were exposed to the ethylene oxide with the treated tubes In each case the control test demonstrated the presence of viable *B. globigii* spores. These tests were performed to show that the amount of ethylene oxide absorbed by each of the tubes was insufficient to suppress *B. globigii* proliferation in the medium.

RESULTS

Table I shows the ratio of the number of tubes which showed no growth of *B. globigii* in the fluid thioglycollate medium to the total number treated The number of viable spores recovered from a representative sample tube prior to treatment is shown next to the test result.

The effect of the two-fold variation in ethylene oxide concentration was negligible What effect there was indicated the higher concentrations to be more efficacious. No attempt was made to control the humidity in the glass flask other than to use pure ethylene oxide vapor and to use standardized amounts of the hygroscopic materials.

Because of the identical treatment for each of the four categories, the fractions of success in sterilization may be compared among the categories Confidence intervals (99%), based on the binomial distribution for each of the four categories, were calculated for the fraction of the tubes sterilized In Table I these confidence intervals (16) indicate that the differences observed in the effect of ethylene oxide on *B. globigii* spores in cases B and C were significantly greater than those in cases A and D Such differences could hardly be ascribed to chance

One might speculate on the cause for such great differences. Both glycerin and paper absorb large quantities of water vapor in comparison with polystyrene and clean glass Even in the environ-

TABLE I.—EFFECT OF INOCULATION METHOD ON EVALUATION

Treatment		Category: A Polystyrene-Distilled Water[a]		B Polystyrene-2% Glycerin[b]		C Filter Paper-Distilled Water[c]		D Glass-Distilled Water[d]	
Absolute Pressure, in. of Mercury	Concentration of Ethylene Oxide, mg./liter	Sterile/Treated	Spores Recovered 1,000's	Sterile/Treated	Spores Recovered 1,000's	Sterile/Treated	Spores Recovered, 1,000's	Sterile/Treated	Spores Recovered, 1,000's
17.24	996	5/10	400	10/10	400	7/10	600	5/10	100
15.90	920	1/10	200	8/10	700	10/10	60	1/10	900
15.41	892	9/9	200	10/10	300	10/10	400	10/10	800
15.25	883	5/5	3	5/5	40	5/5	40	3/4	10
15.14	876	3/5	100	5/5	300	5/5	500	0/5	400
15.00	868	1/5	40	5/5	400	5/5	400	2/5	200
14.43	835	8/10	100	10/10	600	10/10	600	6/10	500
14.08	815	3/10	50	10/10	500	10/10	300	3/10	300
13.59	787	4/10	50	10/10	300	9/10	500	7/10	400
13.48	780	2/10	100	10/10	200	5/10	300	1/10	400
13 07	756	3/10	400	10/10	400	7/10	500	5/10	400
12.96	750	3/10	500	10/10	700	6/10	700	6/10	700
12.96	750	4/10	1000	10/10	3000	6/10	2000	2/10	2000
12.95	749	0/10	500	9/10	700	10/10	700	5/10	800
12.61	730	5/10	40	10/10	500	10/10	400	2/10	500
12.37	716	0/10	300	0/10	300	0/10	400	0/10	500
11.25	650	4/10	300	10/10	400	9/10	400	9/10	400
9.09	525	6/10	100	10/10	600	9/10	400	10/10	100
Totals		66/164	...	152/165	...	131/165	...	77/164	...
Confidence interval (99%)		0.30 to 0.50		0.85 to 0.97		0.72 to 0.88		0.37 to 0.57	

[a] Distilled water suspension of spores was dried on the inside of polystyrene tubes.
[b] Two per cent glycerin suspension of spores was dried on the inside of polystyrene tubes.
[c] Distilled water suspension of spores was dried on filter paper which was inside polystyrene tubes.
[d] Distilled water suspension of spores was dried on the inside of glass tubes.

ment of the 32° incubator, glycerin and paper can absorb appreciable moisture (18) when the ambient relative humidity is 30% or higher. The slow release of this moisture into the ethylene oxide which is initially free of water vapor will maintain high relative humidity in the vicinity of the spore, for at least a portion of the exposure to ethylene oxide. The amount of paper used in each test (10 strips each weighing 10 mg.) was insufficient to affect greatly the humidity of the ethylene oxide (15) remote from the paper. In a sterilizer containing a large quantity of cellulosic materials, the humidity of the ethylene oxide may be quite high after equilibrium is reached. This is particularly true if these materials were in equilibrium with high humidity air before entering the sterilizer. For sterilization, the amount of water vapor which must be added to the gas appears to depend on the amount of water absorbed by the material supporting the bacterial spore. Too much absorbed moisture may strip the ethylene oxide from the gas. Too little moisture in the gas may spare the microorganisms.

Variations in the relative humidity in the vicinity of the spores appear to have a greater effect on the sterilization than do variations in the concentration of ethylene oxide. These factors might have significance with respect to the sterilization process as well as to methods of evaluation.

SUMMARY

Glycerin and/or filter paper in immediate contact with dried *B. globigii* spores reduced their resistance to ethylene oxide exposure significantly. In designing sterilization processes for nonhygroscopic materials and in designing evaluation methods for sterilization of any material, these results indicate how important truly representative inoculation techniques may be.

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Treatment		Category A: Polystyrene Distilled Water[a]		Category B: Polystyrene 2% Glycerin[b]		Category C: Filter Paper Distilled Water[c]		Category D: Glass Distilled Water[d]	
Absolute Pressure, in of Mercury	Concentration of Ethylene Oxide, mg /liter	Sterile/ Treated	Spores Recovered 1,000's	Sterile/ Treated	Spores Recovered 1,000's	Sterile/ Treated	Spores Recovered, 1,000's	Sterile Treated	Spores Recovered, 1,000's
17 24	996	5/10	400	10/10	400	7/10	600	5/10	100
15 90	920	1/10	200	8/10	700	10/10	60	1/10	900
15 41	892	9/9	200	10/10	300	10/10	400	10/10	800
15 25	883	5/5	3	5/5	40	5/5	40	3/4	10
15 14	876	5/5	100	5/5	300	5/5	500	0/5	400
15 00	868	1/5	40	5/5	400	5/5	400	2/5	200
14 43	835	8/10	100	10/10	600	10/10	600	6/10	500
14 08	815	3/10	50	10/10	500	10/10	300	3/10	300
13.59	787	4/10	50	10/10	300	9/10	500	7/10	400
13 48	780	2/10	100	10/10	200	5/10	300	1/10	400
13 07	756	3/10	400	10/10	400	7/10	500	5/10	400
12 96	750	3/10	500	10/10	700	6/10	700	6/10	700
12 96	750	4/10	1000	10/10	3000	6/10	2000	2/10	2000
12 95	749	0/10	500	9/10	700	10/10	700	5/10	800
12 61	730	5/10	40	10/10	500	10/10	400	2/10	500
12 37	716	0/10	300	0/10	300	0/10	400	0/10	500
11 25	650	4/10	300	10/10	400	9/10	400	9/10	400
9 09	525	6/10	100	10/10	600	9/10	400	10/10	100
Totals		66/164	...	152/165	..	131/165	...	77/164	. .
Confidence interval (99%)		0 30 to 0.50		0.85 to 0 97		0 72 to 0 88		0.37 to 0 57	

[a] Distilled water suspension of spores was dried on the inside of polystyrene tubes
[b] Two per cent glycerin suspension of spores was dried on the inside of polystyrene tubes
[c] Distilled water suspension of spores was dried on filter paper which was inside polystyrene tubes
[d] Distilled water suspension of spores was dried on the inside of glass tubes

ment of the 32° incubator, glycerin and paper can absorb appreciable moisture (18) when the ambient relative humidity is 30% or higher The slow release of this moisture into the ethylene oxide which is initially free of water vapor will maintain high relative humidity in the vicinity of the spore, for at least a portion of the exposure to ethylene oxide The amount of paper used in each test (10 strips each weighing 10 mg) was insufficient to affect greatly the humidity of the ethylene oxide (15) remote from the paper In a sterilizer containing a large quantity of cellulosic materials, the humidity of the ethylene oxide may be quite high after equilibrium is reached This is particularly true if these materials were in equilibrium with high humidity air before entering the sterilizer For sterilization, the amount of water vapor which must be added to the gas appears to depend on the amount of water absorbed by the material supporting the bacterial spore Too much absorbed moisture may strip the ethylene oxide from the gas Too little moisture in the gas may spare the microorganisms

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SUMMARY

Glycerin and or filter paper in immediate contact with dried *B globigii* spores reduced their resistance to ethylene oxide exposure significantly *In designing sterilization processes for nonhygroscopic materials and in designing evaluation methods for sterilization of any material, these results indicate how important truly representative inoculation techniques may be*

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depends on the size of the inoculum For example, assume that the probability that a spore selected at random from the inoculum would survive an exposure of t minutes under specified processing conditions is

$$p = e^{-kt} \qquad \text{(Eq 1)}$$

(The constant k has units of reciprocal minutes) One can show that the probability of failing to sterilize the object after an exposure of t minutes under the same processing conditions is approximately

$$P = Ne^{-kt} \qquad \text{(Eq 2)}$$

for large exposure times The number of spores in the inoculum is N To reduce the probability of failing to sterilize to a very low level, either the size of the inoculum must be reduced or the exposure time must be sufficiently increased Equations 1 and 2 have been amply confirmed (2 1, 20) for both ethylene oxide and steam sterilization In practice, N cannot be reduced below the maximum microorganism population which might naturally inhabit the object Because microorganisms have finite volumes, N cannot be infinitely large Usually N is chosen (11, 14) sufficiently large to include an ample safety factor over the natural population size observed to exist in representative samples of similar objects

Some experimenters (5, 20) have used suspensions of spores deposited and dried on the surface to be sterilized Others have used small pieces of paper (11) or fabric (20) which have been inoculated with spore suspensions and dried before insertion into containers to be sterilized Still others have dried suspensions of spores in starch paste or physiological saline, lactose or glycerine solution on the surface to be inoculated

Variations in method of inoculating surfaces to be used in proving sterilization have been significant The effect of such variations was measured in the experiment to be described To the extent possible, the size of the inoculum was uniform and the ethylene oxide concentration was varied in a systematic manner

METHODS

The inside surfaces of tubes one fourth inch in internal diameter and seven-eighths inch in length were inoculated with *B globigii* spores Except for category B, described in Table I, the spores were dried from suspension in distilled water The spores were washed in distilled water before the final suspension was prepared to free them of hygroscopic and other substances which are not part of their innate structure In category B the suspension contained 2% glycerin U S P In category C the spores were deposited on the filter paper only The tubes were inoculated with uniform volumes of the stock spore suspension The number of viable spores recovered by a single rinse was determined by a plate counting technique using a representative inoculated tube after it had dried for twenty-four hours in a 32° incubator While the recovery of spores in categories B, C, and D was roughly the same, relatively fewer were recovered when dried on polystyrene from suspension in distilled water

For each concentration of ethylene oxide shown in Table I the indicated number of tubes representing each inoculation method were placed together in a 200 ml glass flask immersed in an air bath thermostat After evacuation with an oil sealed mechanical pump, the indicated amount of dry pure ethylene oxide vapor was admitted to the flask The concentration was computed from the measured pressure in the flask at 37°, the temperature of the air bath after the system had reached equilibrium Equilibrium was attained in a matter of a very few minutes The ethylene oxide was left in contact with the inoculated tubes for seventeen hours at 37 ± 1° before it was removed by evacuation and replaced with air filtered through a sterile cotton filter

Each of the treated tubes was tested for sterility using procedures described in the "United States Pharmacopeia" (19) Each was placed in a test tube containing 40 ml of fluid thioglycollate medium and incubated there for seven days at 32° In every experiment two untreated but inoculated controls from category D were combined with two polystyrene tubes, which had not been inoculated, in ten ml of the medium The two polystyrene tubes were exposed to the ethylene oxide with the treated tubes In each case the control test demonstrated the presence of viable *B globigii* spores These tests were performed to show that the amount of ethylene oxide absorbed by each of the tubes was insufficient to suppress *B globigii* proliferation in the medium

RESULTS

Table I shows the ratio of the number of tubes which showed no growth of *B globigii* in the fluid thioglycollate medium to the total number treated The number of viable spores recovered from a representative sample tube prior to treatment is shown next to the test result

The effect of the two fold variation in ethylene oxide concentration was negligible What effect there was indicated the higher concentrations to be more efficacious No attempt was made to control the humidity in the glass flask other than to use pure ethylene oxide vapor and to use standardized amounts of the hygroscopic materials

Because of the identical treatment for each of the four categories, the fractions of success in sterilization may be compared among the categories Confidence intervals (99%), based on the binomial distribution for each of the four categories, were calculated for the fraction of the tubes sterilized In Table I these confidence intervals (16) indicate that the differences observed in the effect of ethylene oxide on *B globigii* spores in cases B and C were significantly greater than those in cases A and D Such differences could hardly be ascribed to chance

One might speculate on the cause for such great differences Both glycerin and paper absorb large quantities of water vapor in comparison with polystyrene and clean glass Even in the environ

TABLE I.—EFFECT OF INOCULATION METHOD ON EVALUATION

Treatment		Category							
		A Polystyrene Distilled Water[a]		B Polystyrene 2% Glycerin[b]		C Filter Paper Distilled Water[c]		D Glass Distilled Water[d]	
Absolute Pressure in of Mercury	Concentration of Ethylene Oxide, mg /liter	Sterile/ Treated	Spores Recovered 1,000's	Sterile/ Treated	Spores Recovered 1,000's	Sterile/ Treated	Spores Recovered 1,000's	Sterile/ Treated	Spores Recovered, 1,000's
17 24	996	5/10	400	10/10	400	7/10	600	5/10	100
15 90	920	1/10	200	8/10	700	10/10	60	1/10	900
15 41	892	9/9	200	10/10	300	10/10	400	10/10	800
15 25	883	5/5	3	5/5	40	5/5	40	3/4	10
15 14	876	5/5	100	5/5	300	5/5	500	0/5	400
15 00	868	1/5	40	5/5	400	5/5	400	2/5	200
14 43	835	8/10	100	10/10	600	10/10	600	6/10	500
14 08	815	3/10	50	10/10	500	10/10	300	3/10	300
13 59	787	4/10	50	10/10	300	9/10	500	7/10	400
13 48	780	2/10	100	10/10	200	5/10	300	1/10	400
13 07	756	3/10	400	10/10	400	7/10	500	5/10	400
12 96	750	3/10	500	10/10	700	6/10	700	6/10	700
12 96	750	4/10	1000	10/10	3000	6/10	2000	2/10	2000
12 95	749	0/10	500	9/10	700	10/10	700	5/10	800
12 61	730	5/10	40	10/10	500	10/10	400	2/10	500
12 37	716	0/10	300	0/10	300	0/10	400	0/10	500
11 25	650	4/10	300	10/10	400	9/10	400	9/10	400
9 09	525	6/10	100	10/10	600	9/10	400	10/10	100
Totals		66/164	. .	152/165	.	131/165	. .	77/164	. . .
Confidence interval (99%)		0 30 to 0 50		0.85 to 0 97		0 72 to 0 88		0.37 to 0 57	

[a] Distilled water suspension of spores was dried on the inside of polystyrene tubes
[b] Two per cent glycerin suspension of spores was dried on the inside of polystyrene tubes
[c] Distilled water suspension of spores was dried on filter paper which was inside polystyrene tubes
[d] Distilled water suspension of spores was dried on the inside of glass tubes

ment of the 32° incubator, glycerin and paper can absorb appreciable moisture (18) when the ambient relative humidity is 30% or higher The slow release of this moisture into the ethylene oxide which is initially free of water vapor will maintain high relative humidity in the vicinity of the spore, for at least a portion of the exposure to ethylene oxide The amount of paper used in each test (10 strips each weighing 10 mg) was insufficient to affect greatly the humidity of the ethylene oxide (15) remote from the paper In a sterilizer containing a large quantity of cellulosic materials, the humidity of the ethylene oxide may be quite high after equilibrium is reached This is particularly true if these materials were in equilibrium with high humidity air before entering the sterilizer For sterilization, the amount of water vapor which must be added to the gas appears to depend on the amount of water absorbed by the material supporting the bacterial spore Too much absorbed moisture may strip the ethylene oxide from the gas Too little moisture in the gas may spare the microorganisms

Variations in the relative humidity in the vicinity of the spores appear to have a greater effect on the sterilization than do variations in the concentration of ethylene oxide These factors might have significance with respect to the sterilization process as well as to methods of evaluation

SUMMARY

Glycerin and/or filter paper in immediate contact with dried *B globigii* spores reduced their resistance to ethylene oxide exposure significantly In designing sterilization processes for nonhygroscopic materials and in designing evaluation methods for sterilization of any material, these results indicate how important truly representative inoculation techniques may be.

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Esters of Erythromycin IV*

Alkyl Sulfate Salts

By V. C. STEPHENS, J. W. CONINE, and H. W. MURPHY

The alkyl sulfate salts of various erythromycin[1] esters were prepared by esterification of erythromycin followed by a double decomposition salt formation. These compounds, and in particular propionyl erythromycin lauryl sulfate, are virtually tasteless and are unaffected by mixing with gastric acid. These properties render them very useful in the preparation of flavored suspensions.

Many salts of erythromycin have been extensively evaluated and a number of them are used commercially The esters of erythromycin, while somewhat weaker bases than the parent antibiotic, form stable salts with relatively strong acids, as was previously reported (1) The long chain fatty acids, such as oleic and stearic acid, which have been employed to modify the taste of erythromycin, do not form stable salts with erythromycin esters.

In the last paper of this series (2) it was shown that propionyl[2] erythromycin[3], when ingested by fasting patients, produces antibiotic levels in the blood which are greatly superior to those produced by erythromycin itself. The utilization of this more effective compound in other forms of oral medication besides capsules is of great importance In particular, a flavored suspension could be used advantageously in many patients who find it difficult or impossible to swallow a capsule.

The formulation of propionyl erythromycin into such a flavored suspension has been complicated by two of its characteristics. (*a*) Although dry propionyl erythromycin has only a faintly bitter taste, its flavor in an aqueous suspension is very unpleasant; and (*b*) The exposure of propionyl erythromycin, like erythromycin itself, to gastric juice results in a rapid loss in antibiotic activity.

In view of this acid instability of propionyl erythromycin, one can explain its high degree of effectiveness in capsule form by assuming that while the capsule will normally disintegrate within the stomach, the contents are largely protected by a very low wettability until they have passed into the intestine. Lower antibiotic blood levels are obtained when propionyl erythromycin is given with food than when it is administered orally to fasting patients. When a wetting agent is added to the contents of the capsule a markedly lower level is also produced[4] (Fig. 1). A flavored suspension would be completely wet before ingestion and would thus provide a maximum opportunity for contact with the gastric contents

We have prepared and evaluated a number of *salts of propionyl erythromycin and other* erythromycin esters Of the various salt types tested, the alkyl sulfate salts, such as propionyl erythromycin lauryl sulfate[5] (PELS), have been found to be very useful, especially in the preparation of flavored suspensions

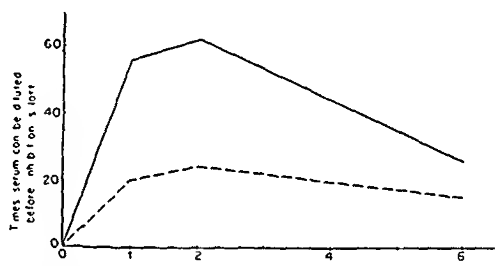

Fig 1 —Crossover blood levels of 10 fasting subjects, 250-mg dose in capsules Solid line, propionyl erythromycin, broken line, propionyl erythromycin plus 5% polyoxyethylene sorbitan monooleate

The chemical structure of PELS is given in Fig. 2. Table I contains a brief summary of the alkyl sulfate salts which were prepared and evaluated. Since they are similar in properties, only PELS will be discussed in detail. PELS is easily prepared from erythromycin by esterification followed by a double decomposition reaction with sodium lauryl sulfate to form the salt The method of preparation is outlined in the experimental section of this report All of the other salts listed in Table I were prepared by the same general method The melting points are not given since all of the compounds melt, with decomposition, at about the same point The exact point of decomposition is somewhat de-

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[1] Ilotycin is the Lilly trade name for erythromycin

The authors wish to express their appreciation to the various members of Eli Lilly and Co who have aided and cooperated in obtaining the data reported

[2] While an "ate" ending is commonly employed for both esters and salts, in this paper the ester group will be named as a prefix and the salt as a suffix, i e , propionyl erythromycin acetate rather than erythromycin propionate acetate

[3] Ilosone is the Lilly trade name for propionyl erythromycin ester.

[4] The blood levels reported were obtained from Dr R S Griffith of the Lilly Clinical Research Laboratories

[5] Ilosone Lauryl Sulfate is the Lilly trade name for propionyl erythromycin ester lauryl sulfate

TABLE I.—PHYSICAL CHARACTERISTICS OF ALKYL SULFATES

X	Y	Assays Erythromycin Theory,[a] mcg./mg	Chem	Biol	SO_4, % Theory[a]	Found
Acetyl	Lauryl	680	664	675	8 9	8 8
Propionyl	Lauryl	674	650	639	8 8	8 7
Acrylyl	Lauryl	675	623	680	8 8	8 7
Crotonyl	Lauryl	669	593	416	8 7	8 9
n-Butyryl	Lauryl	666	597	635	8 7	8 8
Methoxyacetyl	Lauryl	665	637	673	8 6	8 4
n-Valeryl	Lauryl	657	598	635	8 6	8 5
Iso-Valeryl	Lauryl	657	602	562	8 6	8 2
Ethyl carbonyl	Lauryl	665	664	608	8 7	8 5
Ethyl succinyl	Lauryl	630	554	548	8.2	8 7
Propionyl	Octyl	714	676	680	9 3	9 7
Propionyl	2-Ethyl hexyl	714	721	722	9 3	9 0
Propionyl	Tetradecyl[b]	657	636	646	8 6	8 5
Propionyl	Heptadecyl[c]	630	562	572	8.2	8 5
Propionyl	Stearyl	624	613	670	8 1	8 2
Chloroacetyl	Lauryl	661	648	.	3 19[d]	3 04
α-Chloropropionyl	Lauryl	655	605		3 12[d]	2 70

[a] Theoretical values are corrected for about 3% moisture content
[b] 2-Methyl-7-ethyl undecyl-4-sulfate.
[c] 3,9-Diethyl tridecyl-6-sulfate
[d] Chlorine analyses Theoretical content corrected for moisture.

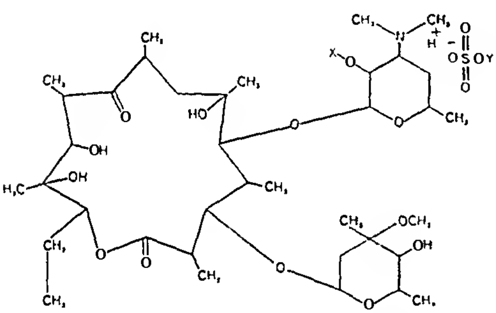

Fig. 2—Structure of acyl erythromycin alkyl sulfates. For PELS, X is $CH_3CH_2C(=O)$—; and Y is $C_{12}H_{25}$.

pendent on the rate of heating. PELS melts, with decomposition, between 135–140°.

PELS labeled with S^{35} was prepared as outlined in the experimental section in about 90 per cent yield.

The water solubility of PELS is extremely low. By the usual physiochemical measurements, only an approximate water solubility could be obtained. By assaying a saturated aqueous solution for erythromycin content by the ultraviolet method commonly employed (3), the water solubility was found to be about 0.024 mg. per cc. This is approximately one-twelfth the solubility of propionyl erythromycin and one-fortieth that of erythromycin. With this very low solubility it is not surprising that the compound is substantially tasteless. PELS can be readily formulated into a pleasantly flavored aqueous suspension.

As was previously mentioned, when propionyl erythromycin and erythromycin are mixed with pooled human gastric juice they dissolve and their antibiotic activity is quickly lost. Salts of carboxylic acids, even though they are water insoluble, behave similarly. The strong acid of gastric juice liberates the free carboxylic acid and dissolves the antibiotic as the soluble hydrochloride salt. PELS, however, is a salt of a very strong acid (lauryl sulfuric acid). The gastric acid is not strong enough to displace the acid radical of the salt and, when mixed with gastric juice, it remains undissolved and retains its potency even when exposed to the acid for extended periods (Table II). The blood levels produced by PELS are not significantly different in the fasting and nonfasting subject (Fig. 3). If anything, the peak level is attained somewhat sooner when the antibiotic is ingested with food. This may be due to a greater dispersion of the drug particles or to an increased production of digestive fluids.

TABLE II.—GASTRIC JUICE STABILITIES

Compound	Exposure Time, min	Activity Retained at 37°, %
Erythromycin[a]	5	3.5
Ethyl carbonyl erythromycin[a]	5	5 7
Erythromycin stearate[a]	5	2
Propionyl erythromycin[b]	5	5 4
Propionyl erythromycin saccharinate[b]	15	10
Propionyl erythromycin lauryl sulfate[b]	40	97.3

[a] pH 1 28 [b] pH 1 10

Recent work reported by Schanker, *et. al* (4, 5), on the mechanism of drug absorption has shown that organic compounds are probably absorbed in the unionized form. It was stated in

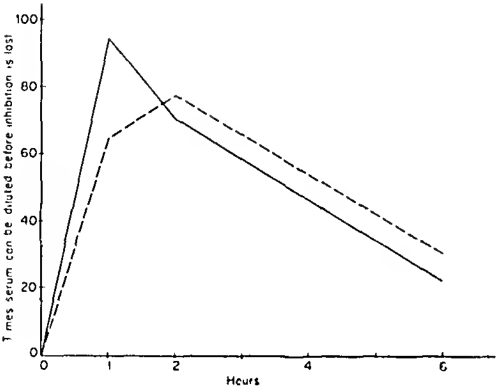

Fig 3—Crossover blood levels for 10 subjects, 250-mg dose of a flavored suspension of PELS Solid line, with food; broken line, without food

the first of these reports (4) that basic drugs having pKa's of less than 8 were readily absorbed while stronger bases were absorbed very slowly. If this proposed mechanism is valid for erythromycin and its derivatives, the greater absorption of the propionyl ester (pK'a 6.9) as compared to erythromycin (pK'a 8.6) may be readily explained.

The use of a salt such as PELS would not be expected to affect this absorption process markedly. In a buffered system, such as the intestinal contents, the ratio of ionized to unionized material in solution would be dependent on the pH rather than on the form administered. Since both propionyl erythromycin and PELS probably pass into the intestine largely in the solid state, their relative solubilities in water might exert some influence on the rates of absorption. Our very limited experience with an aqueous suspension of propionyl erythromycin and our previous work with ethyl carbonyl erythromycin have indicated that both of these compounds are more quickly absorbed than PELS, but the latter appears to give a somewhat longer effective level.

Blood samples from subjects who received propionyl erythromycin, as the base or as PELS, were extracted with chloroform and the antibiotic factors separated by paper chromatography. In every case the blood contained both erythromycin and its propionyl ester. No significant difference appeared as a result of the form of the drug administered. The ratio of esterified to free erythromycin varies from subject to subject and according to the interval between ingestion and blood withdrawal.

The very poor blood levels obtained with erythromycin esters in which the acid radical contains five or more carbons (2) are not readily explained by this mechanism.

EXPERIMENTAL

Propionyl Erythromycin Lauryl Sulfate (PELS) from Propionyl Erythromycin.—Propionyl erythromycin (32.6 Gm.) was dissolved in 300 cc. of anhydrous methanol-free acetone After filtration, a filtered solution of purified sodium lauryl sulfate (12.8 Gm.) in 200 cc. of distilled water was added. A solution of 4.4 cc. of glacial acetic acid in 20 cc. of water was then introduced, followed by another 175 cc. of water which was added rapidly while stirring. The desired salt formed very quickly and was removed by filtration It was washed several times with water and dried in a vacuum oven at 50°. The yield of long white needles was 41.5 Gm.

PELS from Erythromycin.—Propionic anhydride (85 cc.) was added to a solution of 395 Gm. of erythromycin dissolved in 1,500 cc. of anhydrous methanol-free acetone. It was stirred briefly, filtered, and allowed to stand for two hours at room temperature. By the procedure outlined above, the ester solution was utilized directly to form the lauryl sulfate salt. In this case no additional acid is needed since the excess propionic anhydride and the propionic acid formed in the esterification convert the propionyl erythromycin to the ionic form (propionyl erythromycin propionate). The yield was 555 Gm or about 95%.

Propionyl Erythromycin Lauryl S^{35} Sulfate[6].—By the general procedure given, 430 mg. of erythromycin was esterified and reacted with 145 mg of sodium lauryl S^{35} sulfate containing 2.5 mc. of S^{35}. The yield was 460 mg. of PELS having a specific activity of 6 μc per mg

Stability Study in Pooled Human Gastric Juice.—Pooled normal gastric juice (50 cc) was warmed to 37–39° in a constant temperature water bath and 100 mg of propionyl erythromycin (or other compound) was added. The mixture was stirred constantly. Samples for analysis (1 cc. each) were removed at different time intervals and were quickly transferred to 10-cc. volumetric flasks containing 30 mg. of sodium bicarbonate in 2 cc of water. It was mixed immediately. To each flask 4 cc. of methanol was added, and then sufficient distilled water to give 10 cc. This solution was allowed to stand eighteen hours at room temperature then assayed microbiologically for erythromycin activity.

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[6] Prepared in cooperation with Robert E McMahon, Organic Chemical Division, The Lilly Research Laboratories

Pharmacology and Toxicology of Propionyl Erythromycin Ester Lauryl Sulfate*

By R. C. ANDERSON, C. C. LEE, H. M. WORTH, and P. N. HARRIS

Clinical usage of erythromycin[1] and propionyl erythromycin ester[2] has confirmed the low toxicity in experimental animals as reported from these laboratories. Propionyl erythromycin ester lauryl sulfate[3] (PELS) has been found to have very low water solubility and is easily usable in pleasantly flavored suspensions. Chronic toxicity studies in rats and dogs have demonstrated the absence of visceral or hematopoietic damage following large doses for over three months. Oral administration of PELS produced higher serum concentrations in rats than propionyl erythromycin. Both antibiotics are mainly absorbed from the intestine of rats and excreted in very small quantities in the bile. High concentrations of erythromycin activity were found in the lung, spleen, liver, kidney, and heart after oral administration. The results of absorption studies using S^{35} labeled PELS will be discussed.

CLINICAL EXPERIENCE has confirmed the efficacy and low toxicity of erythromycin since it was discovered in 1952 by McGuire, *et al.* (1), of these laboratories. Various salts and esters have been used in different pharmaceutical forms. The crystalline base has been used in ointments and tablets coated with cellulose acetate phthalate (CAP). Erythromycin ethyl carbonate has been employed in drops or suspensions for pediatric therapy. Erythromycin glucoheptonate has been prescribed for intravenous injection. Extensive research revealed that certain esters, when administered orally in capsules, gave earlier, higher, and more consistent blood levels than were obtained with the base or with CAP-coated tablets. Since our publication of the pharmacology and toxicology of propionyl erythromycin (2), Stephens, *et al.* (3), found that certain unique properties of the lauryl sulfate salt of propionyl erythromycin make it possible to prepare a tasteless suspension which produces high and prolonged blood levels. The pharmacological and toxicological studies of this antibiotic are summarized in this report.

MATERIALS AND METHODS

Propionyl erythromycin ester lauryl sulfate (PELS) assayed 650 mcg. of erythromycin activity/mg. Propionyl erythromycin assayed 880 mcg. of erythromycin activity/mg. These antibiotics were given by weight or by erythromycin activity as stated in different sections of this report.

Acute Toxicity.—PELS, suspended in a 5% solution of gum acacia, was given orally to 40 albino mice and 10 albino rats and subcutaneously to 10 albino rats. All animals were fasted overnight and observed for seven days. Six mongrel dogs were given single doses in capsules and observed for any side effects.

Chronic Toxicity.—*Rats.*—PELS was administered to rats by the drug-diet method described by Anderson, *et al.* (4). A total of 60 albino rats, weighing 80 to 100 Gm., was used. Five groups of 12 each, equally divided as to sex, were fed diets containing 0, 0.05, 0.1, 0.25, and 0.5% of PELS, respectively. These concentrations were equivalent to 0, 0.031, 0.062, 0.155, 0.31% of erythromycin. Food and water were available *ad libitum* and the daily food intake was recorded.

Dogs.—Eight pure-bred beagle hounds, weighing between 8.3 and 13.7 Kg., were given PELS orally in capsules at dose levels of 50–100 mg./Kg. (31–62 mg. of erythromycin activity/Kg.) in two divided doses daily including Saturdays and Sundays. The first dose was administered at 8:00 a.m., the second six to seven hours later. From the twenty-second day a pharmaceutical formulation was used in capsules each of which contained 250 mg. of erythromycin activity. Two to four capsules (36–137 mg. of erythromycin activity/Kg., equivalent to 58–221 mg. of PELS by weight/Kg.) were given daily in divided doses. Careful observations were made for side effects. Blood and urine specimens were collected periodically for various analyses. Blood concentration of the antibiotic was determined at various intervals following the morning dosage. After seventy days of treatment, four dogs were sacrificed and examined grossly. Tissues from the heart, lungs, liver, spleen, kidneys, gastrointestinal tract, and thymus, thyroid, pancreas, and adrenal glands were fixed and stained for microscopic studies. The remaining four dogs continue on test.

Pharmacodynamics.—Three mongrel dogs were anesthetized with sodium phenobarbital, 150 mg./Kg. by vein. The carotid artery was cannulated and blood pressure recorded with a mercury manometer. A cannula was also placed in the trachea and respiration recorded with a tambour through a Haley respirometer. A balloon was placed in the duodenum to record intestinal movements. Electrocardiograms were from the standard Lead II and recorded with a Grass polygraph. PELS, as a 10% suspension in 5% acacia, was injected through a catheter opening into the intestine distal to the balloon.

* Received August 21, 1959, from The Lilly Research Laboratories, Eli Lilly and Co. Indianapolis 6, Ind.
The authors wish to thank members of the Biochemical, Physicochemical, and Pharmacological Divisions of the Lilly Research Laboratories for their assistance during various phases of these studies.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August, 1959.
[1] Ilotycin is the Lilly trade name for erythromycin.
[2] Ilosone is the Lilly trade name for propionyl erythromycin.
[3] Ilosone Lauryl Sulfate is the Lilly trade name for propionyl erythromycin ester lauryl sulfate.

Absorption, Distribution, and Excretion.—*Rats* —One hundred and fifty male albino rats, weighing 125 to 150 Gm , were fasted overnight A single dose of 25 mg /Kg of propionyl erythromycin based on the antibiotic activity was administered orally to 75 rats in the form of a 1% water suspension The remaining 75 rats received an equivalent dose of PELS Groups of 15 rats each were decapitated at one-half, one, two, four, and six hours and the serum from each rat was assayed for antibiotic activity

Twenty-four fasted albino rats, weighing 200 to 225 Gm , were anesthetized with 150 mg /Kg of phenobarbital sodium intraperitoneally A double ligature was placed at the junction between the stomach and the duodenum through a medial incision The bile duct of each rat was cannulated with polyethylene tubing, which was brought out through the incision A single dose of 100 mg /Kg of PELS, as a 2% water suspension, was introduced into the stomach of 12 rats with a 22-gauge needle The remaining 12 rats received the same dose intraduodenally The incision was closed with wound clips and the bile was collected in graduated centrifuge tubes At the end of two hours all rats were decapitated Antibiotic activity was assayed on the serum and the bile appropriately diluted with saline

The bile duct of 16 fasted albino rats, weighing 225 to 250 Gm , was cannulated with polyethylene tubing under ether anesthesia The tubing was brought out through the right posterior abdominal wall and the incision was sutured Ten of these rats were given orally a single dose of 100 mg /Kg of PELS and six rats received the same dose of propionyl erythromycin They were kept in Bollman's restrictive cages (5) The bile was collected in graduated centrifuge tubes at intervals and assayed for erythromycin activity Food and physiological saline were supplied *ad libitum*

To study blood partition of this antibiotic, 20 female albino rats, weighing 200 to 225 Gm , were fasted overnight A single dose of 100 mg /Kg of PELS as a 2% water suspension was given orally At the end of one hour and four hours 10 rats each were anesthetized with ether and blood was drawn from the abdominal aorta and heparinized Erythromycin assays were made on the hemolyzed whole blood, on the hemolyzed saline-washed cells, and on the plasma The hematocrit was also determined

Tissue distribution was studied in 40 female albino rats, weighing 200 to 225 Gm They were fasted overnight A single dose of 100 mg /Kg of PELS as 2% water suspension was given orally to 20 rats and the same amount of propionyl erythromycin to the remaining 20 rats Groups of 10 rats each were decapitated at the end of two hours and seven hours after administration Blood samples were collected separately and the serum was assayed for antibiotic activity The spleen, kidneys, brain, and a sample of about 0 5 Gm of the liver and 0 5 Gm of the lung were removed immediately and homogenized in a glass homogenizer with three volumes of 0 2 *M* phosphate buffer (pH 7 2) The heart was cut into small pieces and homogenized with five volumes of the buffer All tissue homogenates were well shaken before assay for erythromycin activity Assays of tissue homogenates from control rats receiving no drug revealed no erythromycin activity When known quantities of PELS or propionyl erythromycin were added to the various tissues, the recovery of antibiotic activity varied from 64–84% No correction was made to compute the tissue concentration of erythromycin from treated rats

Dogs —Four female mongrel dogs with duodenal fistulas, weighing between 12 2 and 15 7 Kg , were fasted for twenty-four hours A single dose of 25 mg /Kg of PELS in capsule was administered orally or intraduodenally through the fistula, followed by 30 ml /Kg of water through a stomach tube At intervals blood samples were drawn from the jugular vein Assays were made on the serum and on the urine appropriately diluted with saline

Absorption of PELS-S^{35} and Sodium Lauryl Sulfate-S^{35}.—The PELS S^{35} was prepared by Stephens, *et al* , of these laboratories (3) Each mg of PELS S^{35} contained 6 μc of radioactivity About 10 μc of this material plus 16 mg of "cold" PELS were administered orally to eight albino rats, weighing between 180 to 220 Gm , as a suspension in 2 ml of water Four rats were killed by ether inhalation at one hour and the remaining four at four hours All blood was withdrawn from each animal by heart puncture The entire gastrointestinal tract, from the esophagus through the rectum, the liver, and kidneys, was removed All tissues and the blood were digested in warm 1 *N* KOH in ethanol, and counted in Diotol (6) with a tri carb liquid scintillation counter (Packard Instrument Co , LaGrange, Ill , Model 314)

Another group of eight rats was given 2 ml of a sodium lauryl sulfate S^{35} solution which contained 4 4 μc /ml The specific activity was 81 5 μc /mg The gastrointestinal tract, the liver, the kidneys, and the blood were removed, digested, and examined in the same manner as the tissues of the previous group of rats treated with radioactive antibiotic

Microbiological Assay.—Erythromycin activity was determined by an adaptation of the Food and Drug Administration *Sarcina lutea* cup plate assay (7) in undiluted serum, in urine and bile appropriately diluted with physiological saline, in hemolyzed whole blood and in hemolyzed washed red blood cells, in plasma, and in various tissue homogenates All findings were compared against a standard sample of erythromycin base Horse serum, hemolyzed whole blood and hemolyzed washed red blood cells of rats, rat plasma, and 0 2 *M* phosphate (pH 7 2) were used for setting up the standard curves for the appropriate determinations It was found that horse serum, dog serum, and rat serum gave the same standard curves

RESULTS

Acute Toxicity.—The LD_{50}'s of PELS in mice after oral and in rats after oral and subcutaneous administration were found to be greater than 6 45 Gm /Kg During the observation period all animals appeared to be completely normal Previous work from these laboratories reported the LD_{50} of propionyl erythromycin by oral route to be 2 87 Gm /Kg for the mouse and >5 Gm /Kg for the rat (2) Thus, PELS is less toxic to the

mouse and no more toxic to the rat than propionyl erythromycin. The results in dogs are shown in Table I. The incidence of vomiting was much less frequent than has been reported previously with propionyl erythromycin (2).

TABLE I.—TOLERATED DOSES OF PELS IN DOGS

Dog Number	8:30 a. m. Dose mg./Kg.	Effects Before 1:30 p. m.	1:40 p. m. Dose mg./Kg.	Effects Before 4:15 p. m.
3,362	25	No action	200	No action
1,775	25	No action	200	3:00 p. m. Vomited
262	50	No action	50	No action
4,524	50	No action	50	2:23 p. m. Vomited
4,514	100	No action	100	No action
4,526	100	No action	100	No action

Chronic Toxicity.—*Rats.*—The mean growth curves for the female rats fed various concentrations in the diet are found in Fig. 1 and for males in Fig. 2. Growth on the 0.05% diet was similar to the control, whereas rats fed 0.1 to 0.5% of PELS did not grow so rapidly as those fed control diets. The average daily food intake was somewhat less in groups showing a lower growth curve. After eighty-one days, half of the rats in each group were sacrificed and submitted to necropsy. Gross and microscopic examination of the heart, lungs, liver, spleen, kidneys, gastrointestinal tract, thymus, thyroid, pancreas, adrenals, salivary glands, and mesenteric lymph nodes revealed no apparent abnormalities. Terminal blood counts were within normal ranges. Terminal blood levels of this antibiotic showed higher values in rats fed higher concentrations. The other 30 rats remain on test.

Dog.—The body weight of all dogs remained relatively constant throughout the experiment. No apparent side effect was observed in any dog. In the four sacrificed animals, bone marrow, erythrocyte, leukocyte and differential counts, and hematocrit and hemoglobin determinations remained within normal ranges. Whole blood clotting time and clot retraction time did not change in any dog. Blood sugar and nonprotein nitrogen values showed the usual variations. No glycosuria was noted. Albuminuria was seen so rarely and was of such low degree as to be considered within normal expectation in this species (8).

Serum levels of this antibiotic were determined on these dogs at seventeen, fifty-one, and sixty-five days. All eight dogs showed significant concentrations following administration of the drug. Approximately eighteen hours after the last dose, the serum contained no detectable amount of this antibiotic.

Pharmacodynamics.—Three dogs received 36 and 72 mg./Kg. of PELS (equivalent to 25 and 50 mg./Kg. of propionyl erythromycin). In two of the animals the blood pressure began to fall one and one-half to two hours after 36 mg./Kg. to 78 to 81% of the normal rate and remained at this level for more than three hours. The third dog showed no significant change. Following 72 mg./Kg., one dog had no change in blood pressure, one showed an increase of 18%, and the third a fall of 22%.

In two of the three dogs the heart rate was increased by 10–15 beats per minute following 36 mg./Kg. The third dog showed no change. The increase in heart rate followed the fall in blood pressure and was probably compensatory in nature. All three animals had an increase in heart rate following 72 mg./Kg. This varied from 10–15 beats per minute. No significant changes were noted in the configuration of the electrocardiogram after either dose.

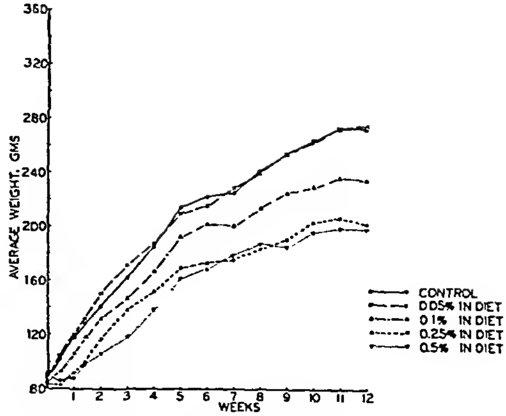

Fig. 1.—The growth curves of female rats fed diets containing PELS.

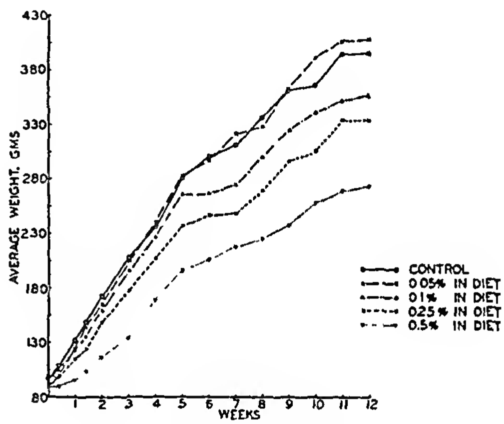

Fig. 2.—The growth curves of male rats fed diets containing PELS.

None of the animals showed significant changes in the respiratory rate following either 36 or 72 mg./Kg. Intestinal motility was stimulated in two dogs after 36 mg./Kg. and in all dogs after 72 mg./Kg.

Absorption, Distribution, and Excretion.—*Serum Levels.*—The serum levels in rats receiving 25 mg./Kg. of erythromycin propionate or of PELS orally are shown in Fig. 3. The serum levels of antibiotic activity produced by PELS were much higher than those obtained with propionyl erythromycin. A peak of serum concentration in rats receiving PELS was reached after two hours, while the propionyl erythromycin produced a maximal serum concentration after one hour. At the end of six hours following administration there were significant amounts of antibiotic in the serum of rats receiving PELS. On the other hand, only a trace of anti-

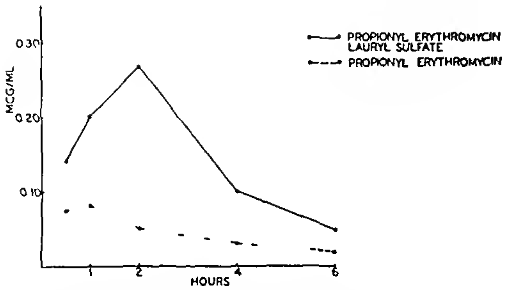

Fig 3.—Serum concentration of erythromycin in rats after oral administration of PELS or propionyl erythromycin, 25 mg. of erythromycin activity/Kg.

biotic activity was found in the serum of rats receiving the propionyl erythromycin.

The average serum concentrations of erythromycin in four dogs with duodenal fistula are summarized in Table II. The serum concentration of antibiotic activity reached a peak two hours after oral administration of 25 mg./Kg. of PELS in capsule. Intraduodenal administration of the same does to the same dogs produced earlier and slightly better serum levels than were obtained by oral administration.

Site of Absorption.—As shown in Table III, when the rat's stomach was ligated at the junction with the duodenum, administration of PELS directly to the stomach resulted in no detectable amount of antibiotic activity in the serum at the end of two hours. Intraduodenal administration produced serum concentrations in all animals.

Urinary Excretion.—Four dogs with duodenal fistula excreted an average of 0.61% of orally administered PELS in six hours. An average of 0.59% of the dose was excreted in the urine when this antibiotic was given to the duodenum through the fistula.

Biliary Excretion.—Results in Table III show that only 0.07% of the administered dose was excreted in the bile of rats in two hours when PELS was introduced into the duodenum that was ligated at the junction with the stomach. The bile concentration of erythromycin averaged 2.1 mcg./ml. These rats excreted an average of 0.02% of the antibiotic when it was administered into the stomach, although no detectable amount of the antibiotic was found in the serum at the end of two hours.

As shown in Table IV, an average of 1.54% of the orally administered dose of PELS was recovered in the bile of rats after twenty-four hours, whereas an average of only 0.91% of propionyl erythromycin was excreted.

Blood Partition.—After oral administration of PELS, erythromycin was found to adsorb on, or penetrate, the red blood cells. After they were washed with saline, the red blood cells contained no detectable amount of erythromycin.

Distribution.—These results are summarized in Table V. In rats, a high concentration of erythromycin was found in the lungs, spleen, liver, kidneys, and heart. After administration of PELS, the erythromycin in the various tissues decreased more slowly than in tissues of rats receiving propionyl erythromycin. Brain tissue contained small amounts of erythromycin after administration of PELS. The

TABLE II.—AVERAGE SERUM CONCENTRATION OF ERYTHROMYCIN (MCG./ML.) IN FOUR DOGS WITH DUODENAL FISTULA AFTER ADMINISTRATION OF PELS

Route of Administration	Time in Hours ½	1	2	3	4	5	6
Oral	<0.03	0.04	0.10	0.08	0.05	0.03	0.03
Intraduodenal	0.08	0.12	0.12	0.07	0.06	0.03	0.03

TABLE III.—BILIARY EXCRETION OF ERYTHROMYCIN IN ANESTHETIZED RATS[a] TWO HOURS AFTER ADMINISTRATION OF PELS

Route of Administration	Serum Concn. mcg./ml.	Bile Volume ml.	Bile Concn. mcg./ml.	% of Dose
Stomach	<0.03	1.78 (1.4–2.0)	2.1 (1.5–6.0)	0.02
Duodenum	0.073[b] (0.04–0.11)	1.90 (1.5–2.4)	6.8 (4.6–10.2)	0.07

[a] The duodenum was ligated at the junction with the stomach.
[b] Numbers in parentheses are the individual ranges within the group.

TABLE IV.—BILIARY EXCRETION OF ERYTHROMYCIN IN RATS AFTER ORAL ADMINISTRATION

Erythromycin	No. of Rats	Per Cent of Dose 0–7 hr.	8–24 hr.	Total
PELS	10	0.55 ± 0.07	0.99 ± 0.16	1.54 ± 0.17
Propionyl erythromycin	6	0.45 ± 0.10	0.46 ± 0.33	0.91 ± 0.17

amount of erythromycin in the brain was about 30 to 40% of the serum concentration.

Absorption of PELS-S^{35} and Sodium Lauryl Sulfate-S^{35}.—Preliminary studies indicated that there was a very small amount of radioactivity in the blood following oral administration of PELS-S^{35}. Based on a blood volume of 5.18%, the total radioactivity in the circulation amounted to less than 1% of the dose. The total radioactivity in the liver and kidneys was even less. Most of the radioactivity was recovered in the gastrointestinal tract and some was excreted in the urine.

After oral administration of sodium lauryl sulfate-S^{35}, slightly more radioactivity was found in the blood, the liver, and the kidneys than in those tissues of rats receiving PELS-S^{35}. However, the radioactivity disappeared somewhat faster from tissues of rats given the first mentioned compound. Again most of the radioactivity was found in the gastrointestinal tract. Further studies are continuing.

DISCUSSION

Rats which were fed PELS in concentrations equivalent to 50 mg./Kg./day for a period of three months showed normal weight gains, while doses of 100–500 mg./Kg./day produced some retardation of growth but no visceral changes attributable to the antibiotic. It is possible that the decreased food intake which accounted for the retardation of growth was a result of lack of palatability. Terminal serum

TABLE V.—TISSUE DISTRIBUTION OF ERYTHROMYCIN MCG./GM. OF WET WEIGHT

	Propionyl Erythromycin		PELS	
	2 hr.	7 hr.	2 hr.	7 hr.
Serum[a]	0.62(0.40–0.85)[b]	0.30(0.13–0.57)	0.68(0.52–1.00)	0.41(0.29–0.60)
Liver	4.43(2 3 –5.8)	1.83(0.9 –3.6)	4.43(3.0 –6.0)	2.89(2.0 –4.2)
Spleen	4 79(2 9 –6.6)	2.76(1.3 –4.6)	4.30(2.2 –6.3)	3.29(2.0 –5.6)
Kidneys	3.59(2.6 –4.0)	1.11(0.5 –2.1)	3.07(1.4 –4.4)	1.91(1.1 –3.1)
Lung	7.50(4.8 –10.8)	3.54(2.1 –5.4)	5.79(4.0 –7.2)	5.53(4.0 –6.8)
Heart	2.36(2.0 –3.6)	0.74(0.6 –1.1)	3.34(1.6 –5.5)	1.55(0.7 –2.6)
Brain	.. (0.00–0.16)	.. (0.00–0 05)	0.20(0.16–0.32)	0.17(0.12–0.28)

[a] mcg./ml. [b] Numbers in parentheses are the individual ranges within the group.

concentrations show that the antibiotic was absorbed in proportion to the amount fed.

The changes in blood pressure, intestinal motility, and heart rate were very similar to those noted with erythromycin hydrochloride and propionyl erythromycin. It appears that these effects are due to the erythromycin content of the antibiotic.

In the rat, PELS produced better serum concentrations than propionyl erythromycin when equal amounts of antibiotic activity were given orally (Fig. 3). When equal weights of these two antibiotics were administered, higher serum concentrations were also obtained with PELS (Table V).

In rats, the major site of absorption of PELS is in the intestines. When PELS was administered to the stomach ligated at the junction with the duodenum, a small amount of erythromycin was recovered in the bile (Table III), indicating that the antibiotic was only slightly absorbed from the stomach. In dogs, PELS is also mainly absorbed from the intestines, since it produced earlier and slightly better serum concentrations after intraduodenal administration than after oral dose (Table II). We have observed (2) that erythromycin propionate was absorbed mainly in the intestines of both dogs and rats.

In anesthetized rats, the serum concentrations of erythromycin following PELS were much lower than the levels found in normal rats after similar doses. This indicates that both the anesthesia and the surgical trauma may have affected the gastrointestinal absorption of this antibiotic.

High concentrations of erythromycin were found in the various tissues following oral administration of both PELS and propionyl erythromycin (Table V). The highest concentration, 3.5 to 7.5 times that of the serum concentration, was in the lung. It is not known how much better PELS is absorbed from the gastrointestinal tract. However, its slower disappearance rate from the various tissues and its limited excretion through the bile may be factors in producing high serum concentrations of erythromycin.

Following oral administration of PELS, rats excreted about 1.54% of the dose in the bile in twenty-four hours (Table IV), about 64% of this amount being excreted during the period from the eighth through the twenty-fourth hour. It appears that this antibiotic is absorbed through the intestines for a relatively long period or that it is slowly released from the tissues to the circulation.

After administration of PELS erythromycin penetrated or was adsorbed on the red blood cells. On washing with saline, erythromycin disappeared from the red blood cells. This indicates that erythromycin is only loosely bound to the red cell component or loosely adsorbed on the cells, a factor beneficial to the therapeutic effectiveness of this antibiotic.

The superiority of propionyl erythromycin over erythromycin base when administered orally has been shown in rats by Lee, *et al.* (2), and in human subjects by Griffith, *et al.* (9). The ability of PELS to produce even better blood levels in rats has been reported in these studies. Data have also been presented that show the very low acute toxicity of this antibiotic, as well as its lack of pathogenic effects when administered daily over extended periods to rats and dogs.

Preliminary radioisotope studies with PELS-S^{35} indicated that a significant amount of radioactivity was absorbed from the intestines. A large percentage of the radioactivity remained in the gastrointestinal tract. The quantity of lauryl sulfate in the highest concentration of PELS fed to rats (0.5%) amounts to approximately 0.15% in the diet. In a two-year study Fitzhugh and Nelson (10) have shown the lack of toxic effects from feeding sodium lauryl sulfate at a 1% level.

SUMMARY

1. PELS was found to have very low acute toxicity. The determination of LD_{50}'s in mice, rats, and dogs was impractical from the standpoint of large quantity of drug needed and consequent mechanical problems..

2. Chronic studies have shown that rats fed PELS in their diet at a dose of approximately 50 mg./Kg./day gained weight comparable to normal animals. Higher concentrations resulted in some growth retardation. No visceral or hematopoietic damage was seen in any animals.

3. Dogs tolerated doses of PELS as large as 221 mg /Kg. (equivalent to 137 mg./Kg. of erythromycin activity) daily over extended periods.

4. Oral administration of PELS produced higher serum concentrations in rats than did propionyl erythromycin.

5. PELS, like the propionyl erythromycin, is mainly absorbed from the intestines of rats.

6. After oral administration of PELS, rats excrete the antibiotic in very small quantities in the bile.

7. High concentrations of erythromycin were found in the lungs, liver, spleen, kidneys, and heart after oral administration of PELS. Small amounts of erythromycin were also found in the brain..

8. It is believed that the superiority of PELS in producing high serum concentrations may be

partly due to its limited biliary excretion and its slow rate of disappearance from the various tissues.

9 Part of the blood erythromycin is loosely bound to red blood cell component or adsorbed on the cells.

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Osmotic Concentration and Osmotic Pressure in Injectable Solutions*

By I. SETNIKAR and OLIMPIA TEMELCOU

A method for determining the osmotic pressure of injectable solutions by measuring the variations of the red-cell volume is described. By means of this method, substances of pharmaceutical interest can be classified into different groups according to their diffusibility through the erythrocyte membrane and their action upon it. It was demonstrated *in vitro* and *in vivo* that for many substances the iso-osmotic concentration is not equivalent to the isotonic concentration and that the confusion between iso-osmia and isotonia can have dangerous consequences.

IT IS COMMON KNOWLEDGE that only solutes which cannot pass through a barrier permeable to the solvent can exert an osmotic pressure (1); substances which can pass through the cell membranes cannot, therefore, counterbalance the osmotic pressure exerted by nondiffusible intracellular solutes

Therefore, the osmotic concentration, measured by physical methods based on one of the colligative properties, is an expression of the osmotic pressure only when all the solutes present in solution are nondiffusible through the cell membranes, otherwise a solution found to be iso-osmotic is hypotonic for the cells

This distinction would be of little practical importance were it possible to accept the view of Szekely and Goyan (2) to the effect that, of the substances in pharmaceutical use, those freely diffusible through the cell membranes are exceptional. The researches performed with a hemolytic method by Husa, *et al* (3–8), demonstrate that, on the contrary, many substances in common pharmaceutical use, at a concentration iso-osmotic with blood, cause hemolysis for the very reason that they are unable to counterbalance the intracellular osmotic pressure

While the hemolytic method can demonstrate very clearly the difference between solutes which are diffusible through the membrane of red cells and those which are not, it is not so easy to determine the isotonic concentration because, for this purpose, it is necessary to start from the premise that for all solutes there is a single ratio between isotonic concentration and hemolytic concentration, whereas it has been shown that, on the contrary, this ratio may vary from 1.4 to 3 1 (9)

Efforts have therefore been directed to the search for a method of direct determination of isotonic concentration and it has been found that this could be done fairly simply by means of a suitable modification of the hematocrit method used by Eijkman (10). This paper describes the method employed and the results obtained therefrom.

METHOD

Human blood was drawn from a forearm vein, rabbit blood by cardiac puncture The syringes used for drawing the blood were moistened with an 0 65% (isotonic) solution of NaF containing 5% heparin The blood samples were centrifuged, the separated red cells were added to an equal volume of the solution under examination, and this suspension was centrifuged for thirty minutes at 3,000 r p m in Wintrobe's hematocrit tubes To determine the volume that the red cells would maintain in an isotonic solution, a similar test was performed mixing the red cells with the plasma of the same specimen of blood

RESULTS

NaCl.—The ratio between the volume of rabbit red cells and the concentration of NaCl is given in

* Received July 6, 1959, from the Research Department, Recordati Laboratorio Farmacologico S p A, Milano, Italy

Fig. 1. In contact with plasma, red cells maintained a volume equal to that which they would take up in contact with an 0.93% solution of NaCl. This concentration is, therefore, isotonic with the red cell specimen used.

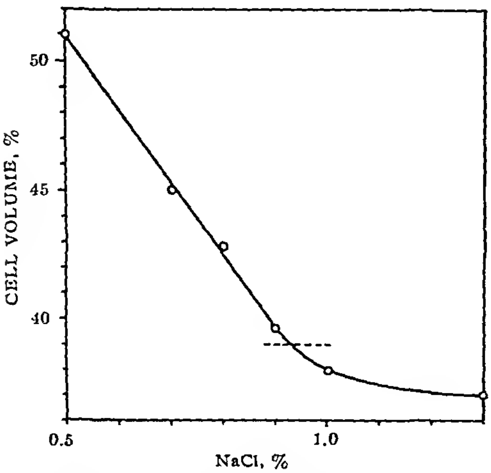

Fig. 1.—Effect of the NaCl concentration upon the volume of rabbit red cells. The dotted line represents the volume of the cells suspended in plasma (isotonic volume). For these red cells the isotonic concentration of NaCl was 0.93%.

Urea.—Solutions of urea from 1 to 2.6% (a 1.8% solution is iso-osmotic) caused complete hemolysis. The addition of NaCl at concentrations from 0.5 to 0.9% to an iso-osmotic solution of urea prevented laking, and the volume of the red cells was equal to that determined by solution of NaCl at the same concentration but without urea. Thus, urea does not of itself have a hemolytic effect, as in the opinion, for example, of Ebina (11); laking is brought about by the incapacity of this substance to counterbalance the intracellular osomotic pressure. In other words, as regards osmotic pressure, it is as if urea were not present in solution.

The incapacity of urea to exert an osmotic pressure can be demonstrated also *in vivo*. If 15 cc./Kg. of a 1.8% solution of urea is injected intravenously into rabbits, extensive hemolysis is observed, due to the destruction of about 3% of the red cells. A similar phenomenon is observed when one administers the same quantity of distilled water. Hemolysis can be entirely avoided by rendering the solution of urea isotonic with a 0.9% solution of NaCl.

Dextrose.—While dextrose exerts an osmotic pressure equal to its concentration on the erythrocytes of rabbits, on human erythrocytes the isotonic concentration is almost twice the iso-osmotic concentration (Fig. 2). The membranes of human erythrocytes would therefore seem to be partially permeable to dextrose. It is interesting to note that the resistance of human red cells increases in solutions of dextrose; the cell volume in hypotonic solutions can attain values practically twice those which usually precede hemolysis.

This increase in cell resistance perhaps accounts for the results obtained by Grosicki and Husa (4), who noted no difference between the

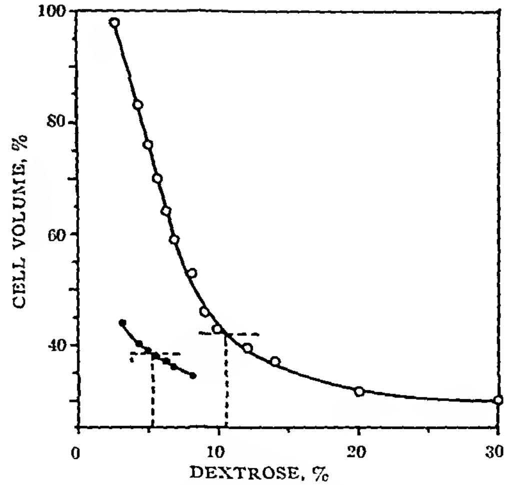

Fig. 2.—Effects of dextrose solutions at different concentrations upon the volume of the red cells of the rabbit and of man. The dotted line indicates the isotonic volume. O—O, human red cells; ●—●, rabbit red cells.

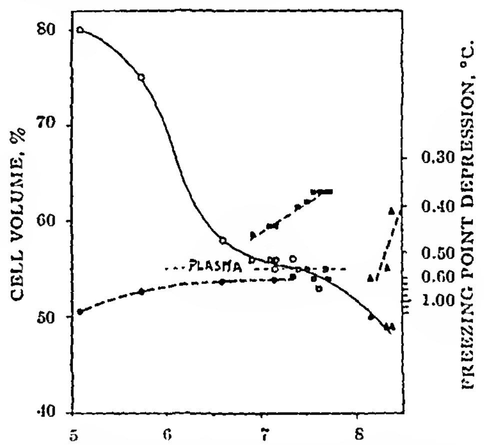

Fig. 3.—Effects of the pH upon the volume of red cells and thus also upon intracellular osmotic pressure. The cations concentration was maintained constant to 155 meq./L. of Na^+.

Left ordinate, cell volume; right ordinate, depression of the freezing point on a scale so adjusted that, for a solution of NaCl, the depressions of the freezing point would correspond to the cell volume; abscissa, pH of the buffer-red cell mixture.

The isotonic cell volume is shown by a dotted line. The straight line and the empty signs represents the cell volume, the dotted line and the full signs give the cryoscopic depression. O—Buffers of acetic acid-sodium acetate, □—buffers of $NaH_2PO_4-Na_2HPO_4$, △-buffers of barbital-sodium barbital. Note the poor correlation between the osmotic concentration measured by the cryoscopic method and the osmotic pressure exerted by the solutions upon the red cells.

hemolytic concentration of dextrose for human red cells and those for rabbit red cells.

Procaine Hydrochloride.—At its iso-osmotic concentration (5.05%), procaine hydrochloride causes hemolysis of the red cells of the rabbit. In contradistinction to what has been observed with urea, to

render the solution isotonic it is not sufficient to add 0 9% of NaCl but it is necessary to add this salt at a concentration of about 1 3%, as if the procaine increased the permeability of the membrane to sodium chloride

It is interesting to note, however, that it is enough to add a 3 3% solution of dextrose (0 6 iso-osmolar) to the same solution of procaine hydrochloride to have a solution which is isotonic for rabbit red cells, as if dextrose not only abolished the permeabilizing effect of procaine but rendered the cell membrane partially impermeable to procaine

Saponin.—This substance has an intense hemolytic effect up to a concentration of 0 005–0 001% even if dissolved in an 0 9% solution of NaCl The behavior of rabbit red cells placed in NaCl and dextrose solutions at different concentrations and in the presence of saponin at 0 05% was checked, and the results were very similar to those obtained with procaine hydrochloride at 5 05%

$ZnSO_4$—Zinc salts are of particular interest inasmuch as Hartman and Husa (6) observed by means of their hemolytic method that $ZnSO_4$ "protects" the red cells to such an extent that the isotonic solution of the salt would be 400 times more dilute than the iso-osmotic concentration Cadwallader and Husa (12) have described similar results for zinc acetate By our method, however, it can be shown that $ZnSO_4$ precipitates plasma proteins and causes hemolysis up to a concentration of 155 m*M* The results described by Hartman and Husa can be confirmed only if blood and $ZnSO_4$ solution are mixed in the volumetric proportion of 1·50 (as these authors did), but it can also be demonstrated that the absence of laking is due to a precipitation and a denaturation of hemoglobin One cannot, therefore, accept the conclusion that solutions of $ZnSO_4$ are isotonic at concentrations 400 times lower than the iso-osmotic concentration because not a protective action but a precipitating and denaturating action by the zinc ion is implicated

Effects of the pH of Solutions on Intracellular Osmotic Pressure.—At physiological pH values negative charges prevail in red cell hemoglobin and about 50 meq /L of cations are required for electrical neutralization As hemoglobin is an ampholyte, its negative charges diminish when the environment becomes acid, releasing cations which, being unable to diffuse in the extracellular fluid through the cell membrane which is impermeable to them, attract anions from the extracellular fluid A diminution of pH, therefore, involves a rise in intracellular osmotic pressure and, conversely, an augmentation of pH causes a fall in intracellular osmotic pressure The isotonic concentration must, therefore, depend to some extent upon the pH of the solution

Figure 3 gives experimental proof of this hypothesis Although the cations concentration of the solution under examination was kept constant (155 meq /L of Na^+), it may be observed that the cell volume increases in acid solutions and decreases in alkaline solutions, demonstrating that the osmotic pressure of red cells increases in contact with acid solutions and decreases in contact with alkaline solutions Here again the osmotic concentration of the various solutions, measured by determining the depression of their freezing points, was not closely related to the osmotic pressure of the solutions

DISCUSSION

Similar experiments carried out on substances of pharmaceutical interest showed that these could be classified into the following groups:

Group 1—Substances whose iso-osmotic concentration is isotonic: NaCl (0.9%), KCl (1 19%), sodium thiosulfate N. F. (2 98%), sodium borate U. S P (2 6%), sodium propionate N F. (1 47%), sodium benzoate U S P. (2 25%), sodium barbital (3 14%), sorbitol (5 48%), and dextrose U. S P. (5 5 %) for rabbit red cells

Group 2—Substances which do not exert any osmotic pressure: urea, succinic dinitrile, antipyrine, aminophylline, ethanol, propylene glycol, sodium pentobarbital, Tween 80

Group 3—Substances whose isotonic concentration is higher than their iso-osmotic concentration· dextrose (as regards human red cells), glycine, sodium salicylate

Group 4—Substances which increase the permeability of the erythrocyte membrane to NaCl· procaine hydrochloride, adiphenine hydrochloride, ethanol, and propylene glycol at higher than 10–20% concentrations, Tween 60

Group 5—Substances with a pronounced hemolytic action: saponin, sulfuric esters of methylandrostenediol and of testosterone

Group 6—Substances which exert a protective action similar to that exerted by dextrose as regards the increase in permeability caused by procaine: dextrose, sorbitol

Group 7—Substances precipitating proteins· $ZnSO_4$ and all precipitants of proteins

SUMMARY

When wishing to render an injectable solution isotonic, the main consideration should be the permeability of the cell membrane to the various solutes composing the solutions and the action of these solutes upon the cell membrane In other words, *iso-osmia* (which can be determined by physical methods based on one of the colligative properties) is equal to *isotonia* only when all the solutes of the solution are unable to diffuse freely through the cell membrane. If this is not the case, isotonia can be determined only by measuring the osmotic effect of a given solution directly upon the concerned cells.

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Polarography of Gibberellic Acid*

By WILLIAM F. HEAD, Jr.

The polarographic characteristics of several gibberellins have been determined and a method for determining gibberellic acid in the presence of gibberellenic acid proposed. Gibberellic acid was found to possess a characteristic prewave in addition to a larger proton reduction wave shown by all gibberellins. This prewave may be measured with an accuracy to ± 2 per cent within definite concentration limits. Average recoveries of gibberellic acid from a typical formulation measured by the method was 97 per cent. The main reduction wave was shown to be that of carboxyl proton but the prewave reduction mechanism is not yet clear.

THE FLUOROMETRIC ASSAY PROCEDURES used for gibberellic acid are dependent upon an effective separation of gibberellic acid from closely related compounds. Potassium carbonate chromatographic columns do not appear to accomplish this separation and other fluorescent, biologically inactive gibberellins are detected as gibberellic acid. The use of ultraviolet irradiation to suppress the fluorescent properties of gibberellenic acid also inhibits the gibberellic acid fluorophor Recently, Kavanagh and Kuzel (1) have described a liquid partition system in which gibberellic acid is successfully separated from gibberellenic acid, a major interfering component.

The time-consuming biological assay (2) is absolute for plant growth activity but rather erratic results have been obtained.

In view of the isolation difficulties, a technique was sought which would allow measurement of gibberellic acid in small quantities in the presence of similar compounds Kitamura and Sumiki (3) have pointed out that certain gibberellins are polarographically reducible, although no indication of specificity was given in their report.

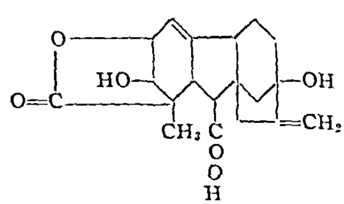
A mass isotope dilution assay based on the isolation of a pure sample of gibberellic acid and its isotope by crystallization has recently appeared (4) This method, advocated for fermentation broths, would not be conveniently applicable to samples containing only microgram quantities.

EXPERIMENTAL

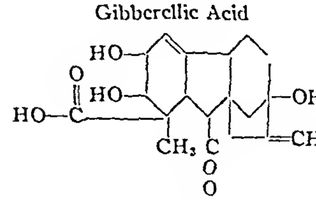
Apparatus and Reagents.—Most polarograms were made using a standard 20-ml capacity H-cell with a dropping mercury electrode, agar-potassium chloride bridge, and a saturated calomel electrode. In some cases a quiet mercury pool was used as an anode in a single chamber cell The polarograph used was a Sargent model XXI All reagents used were analytical reagent grade. In the case of the quaternary ammonium supporting electrolytes, a solution of these reagents showed no polarographically reducible impurities over the voltage range examined.

Polarographic Data.—In Table I is listed the reduction characteristics of several gibberellins All samples included were essentially pure products and a quantitative response was indicated for each reduction wave of all compounds None of these compounds showed reduction waves from −2.0 to −2 7 volts (S. C. E.) when polarographed in a single cell with a quiet mercury pool anode in a 0 1 N tetraethylammonium chloride solution The potentials listed in Table I were all taken in a 0 1 N potassium chloride supporting electrolyte

The data for gibberellic acid were obtained with a working standard containing 94% gibberellic acid (by differential nonaqueous titration and differential fluorescence measurements), 1 5% gibberellenic acid (ultraviolet molecular extinction at 253 $m\mu$ = 19,200), and 4.5% moisture (Karl Fischer). Attempts were made to remove the gibberellenic acid by chromatography through potassium carbonate columns and subsequent elution with 1% water in acetonitrile. After two such treatments, the gibberellenic acid content was reduced to 0 28% and fluorescence in 85% w/w sulfuric acid indicated 99 3% gibberellic acid. When compared polarographically to the initial working standard a purity of only 75% was indicated. Infrared analysis, using the characteristic 5.67 μ lactone band of gibberellic acid and the working standard as a reference, indicated a gibberellic acid content of 82% in the "purified" material. It is thought that lactone hydrolysis may take place to an extent on carbonate columns with the hydrolysis product retaining the fluorescent characteristics of gibberellic acid.



Gibberellic Acid



Hydrolysis Product

* Received July 1, 1959, from the Analytical Research Department, Eli Lilly and Co., Indianapolis 6, Ind.
Dr G W Probst and Dr Koert Gerzon, who supplied the various gibberellins and much background information, are sincerely thanked for their help A debt of gratitude is also due Dr R P Miller and Mr H L Bird, Jr., who chromatographed the various gibberellins during purification and performed the preparative chromatography work

Table I —Reduction Potentials of Several Gibberellins

Compound	$E\,{}^1/_2$ (S C E) Volts
Gibberellic acid	−1 0 to −1 2
	−1 55
Gibberellenic acid	−1 55
Methyl ether of gibberellenic acid	−1 55
Gibberic acid	−1 24
	−1 54
Tetrahydrogibberellic acid	−1 45

No attempts were made to isolate a hydrolysis product and the infrared data are the only evidence in support of such a reaction

In a similar manner, attempts were made to purify gibberellenic acid from a sample containing 15% gibberellenic acid After elution of gibberellic acid with acetonitrile containing 1% water, remaining gibberellins may be eluted with methanol However, this technique resulted in a product containing only 46 7% gibberellenic acid when compared to the pure material at 253 mμ The gibberellic acid polarographic wave at −1 0 to −1 2 v (S C E) was absent Using the top phase of a 1 1 butanol-1 5 *M* ammonia mixture as the developing solvent on Whatman No 1 paper, no other gibberellins could be detected with 0 5% potassium permanganate except gibberellenic acid An attempt was made to purify gibberellenic acid by preparative chromatography in the above solvent system using the techniques of Brownell, Hamilton, and Casselman (6) The final purity of the nonmobile gibberellenic acid was 50 8% This product had the same characteristics as the material purified using the column technique

It is of interest that the 50% gibberellenic acid showed the same i_d/c ratio at −1 55 v (S C E) on a weight basis as did the pure material This points out the carboxylic acid nature of the impurities

Assay Principle.—The polarograms of gibberellic and gibberellenic acid are shown in Figs 1 and 2, respectively It is apparent from Table I that the prewave of gibberellic acid is unique for the gibberellins except for gibberic acid This material is a product of vigorous acid degradation of gibberellic acid (5) and is an unlikely interference in a fermentation broth or a commercial formulation The gibberellic acid prewave at −1 0 v to −1 2 v (S C E) is quantitative, as shown in Fig 3, and its $E\,{}^1/_2$ varies through the range mentioned, becoming more negative with increasing concentration

Gibberellenic acid in the presence of gibberellic acid was shown to have no effect on this prewave The two waves occurring at −1 55 v (S C E) were additive If the total concentration of gibberellins became too large, fusion of the prewave and the additive waves would occur Dilution would again separate the two waves, however, if gibberellic acid was present in very small quantities compared to total gibberellins, accurate measurement of the prewave would be difficult Even with the 94% working standard gibberellic acid, fusion of the prewave can take place with its own reduction wave at −1 55 v (S C E) if the upper concentration limit of Fig 3 is exceeded

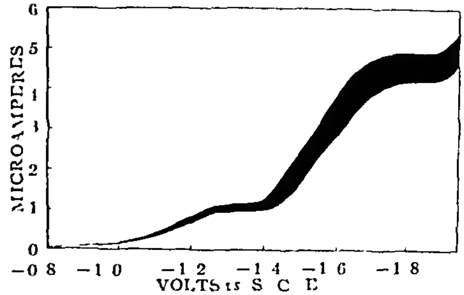


Fig 1 —Gibberellic acid polarogram in *0 1 N* KCl

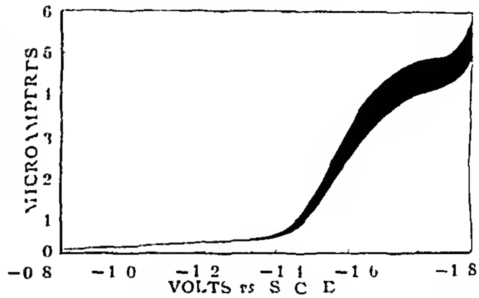


Fig 2 —Gibberellenic acid polarogram in 0 1 *N* KCl

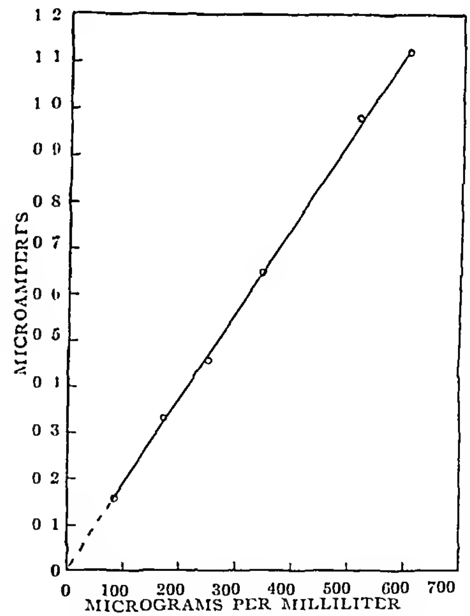


Fig 3 —Gibberellic acid prewave current (i_d) *vs* concentration

Products such as tablets and bulk powders contain biologically inert materials which may interfere with a polarographic assay Sodium lauryl sulfate and various dyes were found to be particularly offensive The surface active agent interfered seriously with the mercury drop rate as well as producing its own reduction wave in the region of the gibberellic acid prewave The dyes, which can be used as maxima suppressors, completely prevented gibberellic acid reduction In

a search for a selective solvent for gibberellins, redistilled methyl isobutyl ketone was found to be satisfactory even though traces of sodium lauryl sulfate were carried along with the gibberellins

Crushed placebo tablet material was extracted in a beaker with redistilled methyl isobutyl ketone The solvent was filtered, evaporated, and the residue dissolved in 0 1 *N* KCl To this solution was added varying amounts of working standard gibberellic acid in order to establish a standard plot of i_d/c It was necessary to use this procedure because the intercept did not occur at the origin due to the small amounts of sodium lauryl sulfate present Using tablets of known gibberellic acid content, an average recovery of 97% was obtained by this method The maximum deviation of the polarographic measurement was observed to be ± 2%

DISCUSSION

Reduction Mechanism.—A plot of i_d/c for the reduction wave at −1 55 v (S C E) of gibberellic acid, shown in Fig 4, immediately suggested a mechanism controlled by the ionization of the carboxyl group Gibberellenic acid was observed to behave in a similar manner These waves are within the range of compounds having reducible functions and one or more carboxylic acid groups

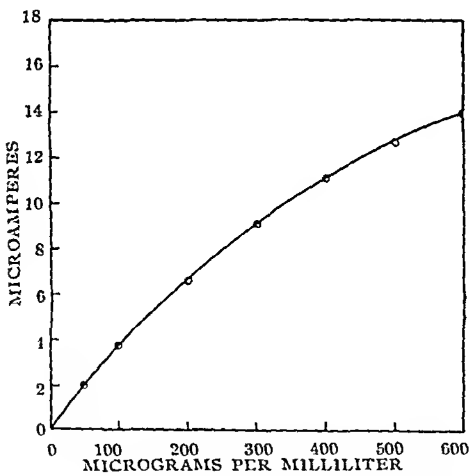


Fig 4 —Concentration *vs* i_d using gibberellic acid wave at −1 55 v (S C. E)

For confirmation of this assumption and in order to study the effects of pH on the prewave, a gibberellic acid solution in 0 1 *N* KCl was polarographed several times after adding small successive increments of 0 002 *N* NaOH The procedure was repeated using 0 002 *N* HCl instead of alkali As seen in Fig 5, both waves of gibberellic acid decrease to zero μa on addition of alkali This zero intercept is equivalent to the titration end-point of the solution As would be expected, the wave at −1 55 v (S C E) increases steadily when the solution is made more acidic. The prewave, however, is relatively unaffected by acid as shown in Fig. 6. Both Figs 5 and 6 are corrected for the effects dilution would have on i_d and represent the effects of acid and alkali concentration only.

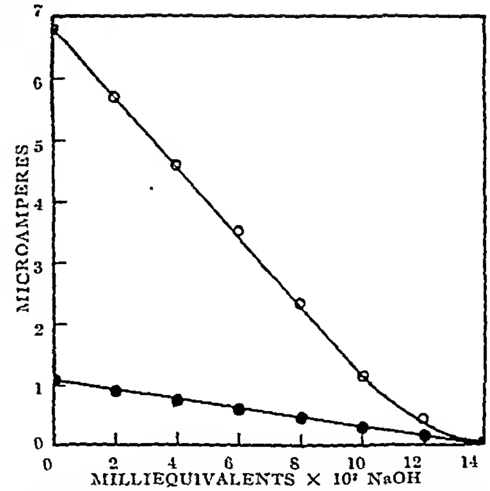


Fig 5 —Effect of alkali on both reduction waves of gibberellic acid O −1 55 v (S C E). ● −1.0 v to −1 2 v (S C E)

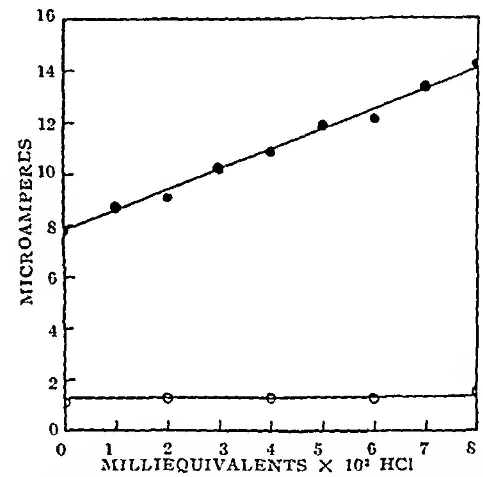


Fig 6.—Effect of acid on both reduction waves of gibberellic acid ● −1 55 v. (S C E) O −1.0 v. to −1 2 v. (S C. E)

A satisfactory explanation of the mechanism of reduction of the prewave is more difficult to obtain Approximation of the Ilkovic equation gave a value for *n* (apparent electron change per molecule reduced) of 0 38 while plots of log $i_d - i/i$ *vs* $E\ ^1/_2$ gave a value of 0.44 This points up the irreversibility of the reduction but the polarographic wave slope is too large to be accounted for by an association or polymerization mechanism (7)

On examination of the structure of gibberellic acid, the only other apparent reducible sites are the methylene group, the lactone group, and the double bond. Since gibberellenic acid contains the same methylene group, it would not be consistent to assign the prewave of gibberellic acid to reduction of this group A similar case may be drawn for the lactone group Tetrahydrogibberellic acid contains the same lactone group as gibberellic acid and is a completely saturated compound It does not show the prewave reduction. In addition, lactones

are not usually reducible at potentials as low as −1 2 v (S C E) (8) By a process of elimination, only the double bond remains as a choice and there has been some question about the polarographic reduction of isolated double bonds (9, 10) Dihydrogibberellic acid (gibberellin A_1) would offer much information about this reduction; however, this compound is not presently available

The effects of pH on the i_d of both waves show that the prewave is not kinetically controlled in a manner similar to pyruvic acid (11) By heating a gibberellic acid solution to 80°, a large increase in i_d was obtained, rather than complete disappearance, indicating that the prewave is not produced by some adsorption phenomenon (12) If a double bond is involved in this reduction, it is possible that a two-step mechanism dependent on proton concentration, as discussed by Elving and Teitelbaum, is operative (13) Such a mechanism would agree well with the observed behavior of gibberellic acid reduction in alkaline media

SUMMARY AND CONCLUSIONS

The polarographic properties of gibberellic acid and some related compounds have been studied. Gibberellic acid reduction is shown to consist of two separate mechanisms giving rise to a normal wave and a prewave. This prewave was found in only one other gibberellin examined and an assay method was based on this characteristic

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Further Studies on the Relation Between *in Vitro* Disintegration Time of Tablets and the Urinary Excretion Rates of Riboflavin*

By A. B. MORRISON, D. G. CHAPMAN, and J. A. CAMPBELL

Rates of urinary excretion of riboflavin were determined after dosing subjects with eight preparations varying in *in vitro* disintegration time and physiological availability. *In vitro* disintegration times were determined by the procedure given in U. S. P. XV Second Supplement, modified by the use of thirty minutes time in simulated gastric fluid and by the use of solid disks. Previous work was confirmed and additional evidence presented to show that tablets which did not disintegrate in one hour by this procedure were not fully available to the body, as judged by the urinary excretion of riboflavin. Use of the fluted disks of the existing U. S. P. procedure did not change the relationship between *in vivo* availability and *in vitro* disintegration time. The height of riboflavin excretion curves was found to be directly proportional to physiological availability of the riboflavin and was inversely related to *in vitro* disintegration time. Since rate of urinary excretion has been shown to be directly related to tissue levels for several drugs, it might be expected that marked differences in blood levels of riboflavin would be produced by the various preparations tested.

The results of earlier studies from this laboratory (1–3) suggested that tablets which did not disintegrate in one hour by a specified *in vitro* test were not fully available to the body, as judged by the urinary excretion of doses of riboflavin and *p*-aminosalicylate Although Endicott and Kirchmeyer (4) questioned these observations, their findings that the riboflavin in tablets disintegrating in thirty-two to forty-one minutes was fully available *in vivo* are in complete agreement with those of Chapman, *et al* (1). Vliet (5) suggested that some of the tablets which Chapman, *et al.* (1), found to have *in vitro* disintegration times of over sixty minutes, may have been enteric coated. In view of these questions, the present studies were conducted to investigate more closely the relationship between availability of riboflavin *in vivo* and the *in vitro* disintegration of sugar coated tablets, particularly those disintegrating within sixty to one hundred and twenty minutes.

* Received June 29, 1959, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada
The authors are grateful for the continued cooperation and assistance of the subjects who took part in this investigation, and are indebted to Mr L G Chatten for some of the *in vitro* disintegration times reported, and to Mr C Perusse for chemical determinations

It has been established that for several drugs, including sulfaethylthiadiazole (6), creatinine (7, 8), xylose (9) and galactose (10), the rate of urinary excretion is proportional to tissue concentration Since the rate of urinary excretion of riboflavin is probably also related to tissue concentration, data were obtained on variation in excretion rate over a period of time after dosing with the various riboflavin-containing preparations

METHODS

Physiological Availability.—As in previous studies (1, 2), the method used to determine the physiological availability of riboflavin was essentially that of Melnick, *et al* (11) Eight sugar coated products were tested during a two-year period. Six to 9 normal male subjects, shown to be receiving nutritionally adequate diets, were used in the experiment While on test, they were allowed to consume their regular meals but were cautioned to refrain from eating foods high in riboflavin, such as liver, and to eat meals similar in nature from day to day. The subjects were given doses of 5 mg or 10 mg of riboflavin in a rapidly disintegrating standard tablet, and 3 mg to 10 mg. of riboflavin as the commercial preparations The doses were given at 8·45 a m after breakfast. Urine was collected in opaque bottles containing 2 ml of 3 5 N H_2SO_4 at two-, four-, six-, eight-, fourteen-, and twenty-four-hour intervals after dosing. Riboflavin in the tablets and urine was determined by the U S P XV fluorometric procedure (12) All gross excretion values were corrected by subtracting the appropriate blank determined on the urine of the same subjects without dosing. Calculations of availability were based on duplicate determinations of riboflavin in the tablets

In Vitro Disintegration Times.—The apparatus described in U S P. XV Second Supplement (13), modified by the use of solid plastic disks, was used to determine disintegration time The tablets were immersed for thirty minutes in simulated gastric fluid and the remainder of the time in simulated intestinal fluid Disintegration times reported were mean times of at least two separate tests on six tablets each.

RESULTS

Descriptive data on the preparations used are summarized in Table I. All of the products were sugar coated multivitamin preparations Products B, C, E, F, and H also contained minerals *In vitro* disintegration times varied from sixty-two minutes (product H) to one hundred and twenty minutes (product D)

The data on urinary excretion of riboflavin after ingestion of 5 mg or 10 mg of the standard dose are summarized in Table 11 The mean excretion in five trials conducted over a two-year period varied from 57 to 60%, with an overall mean of 58% As might be expected in view of the findings of Melnick, *et al* (11), Hegsted, *et al* (14), and others, considerable variation was found in the percentage of the standard dose of riboflavin excreted by the various subjects Significant variation in percentage excretion by the same individual in different trials was also noted The remarkable constancy in the mean percentage excretion observed in repeated trials in the present studies is in agreement with the findings of Melnick, *et al.* (11).

The results of the physiological availability studies are summarized in Table III. The high

TABLE I.—DESCRIPTION OF PRODUCTS STUDIED

Product	Medication	*In Vitro* Disintegration Time, min	Riboflavin/Unit, mg	Riboflavin/Dose, mg.
A	Multivitamins	69	6 0	6 0
B	Multivitamins and minerals	75	5 0	10 0
C	Multivitamins and minerals	75	5 0	5 0
D	Multivitamins	120	4 0	4 0
E	Multivitamins and minerals	78	2 5	5 0
F	Multivitamins and minerals	69	2 5	5 0
G	Multivitamins	69	2 0	4 0
H	Multivitamins and minerals	62	3 0	3 0

TABLE II —PERCENTAGE OF STANDARD DOSE OF RIBOFLAVIN RECOVERED IN URINE

Subject	Trial 1 10 mg Dose 1957	Trial 2 5 mg Dose 1957	Trial 3 5 mg Dose 1959	Trial 4 5 mg Dose 1959	Trial 5 5-mg Dose 1959
A. B M			36	52	72
J A C	58	56	66	64	54
T. K M	38	51	53		75
J M M.	53	49	75		62
R C.	78	76			
D G C	63	62			
L C	56	41			
T P	56			79	
E M.	60	67			
O P	60				
C P			52	27	50
D S			74	50	49
K S			51	67	59
Mean	58	57	58	57	60

TABLE III.—PHYSIOLOGICAL AVAILABILITY OF RIBOFLAVIN IN EIGHT PRODUCTS

Subject	A	B	C	D	E	F	G	H
	Riboflavin Available in Product, %							
A. B M					33	88	11	66
J. A C	103	36	14	5	80	97	35	91
T K M		27	0	11	12	54	38	58
J. M M		80	2	0	69	120	92	98
R C	81	65	48	8		..		
D. G C	46	52	98	0				..
L C		39	2					
T P	42	56						79
E M	98	23	2	22	.	...	..	..
O P.	62	18		53	.	..	.	..
C P	.				19	139	42	..
D S				.	77	53	51	..
K S			.		64	14	45	21
Mean	72	44	24	14	51	81	45	69
In vitro disintegration time								
Solid disks	69	75	75	120	78	69	69	62
Fluted disks	..	.	.	.	89	84	61	60

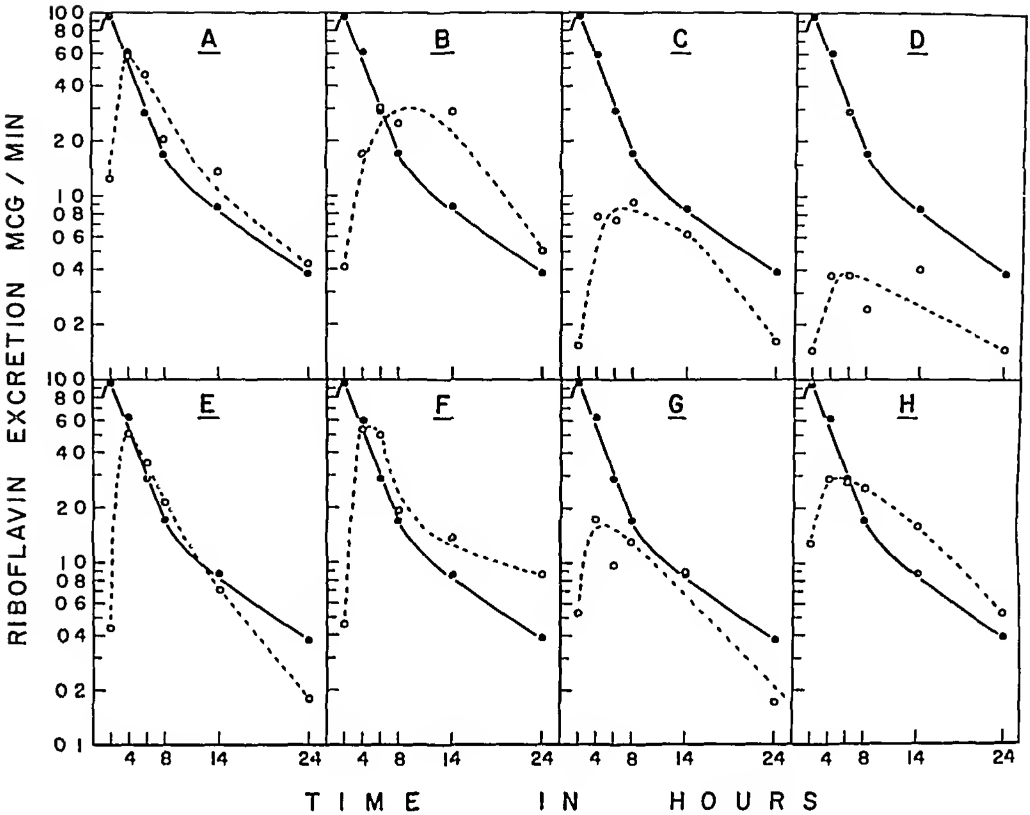

Fig 1 —Urinary excretion curves of riboflavin in eight tablets (O- — -O) disintegrating *in vitro* in one to two hours compared with curves for a riboflavin standard tablet (●——●)

variation noted between subjects is typical of results obtained with preparations on the borderline of availability (1) None of the tablets was fully available physiologically *In vitro* disintegration times tended to be inversely related to physiological availability Preparation A, which disintegrated in sixty two minutes *in vitro*, was only 69% available *in vivo* Preparation D, which had the longest disintegration time studied (one hundred and twenty minutes), was the least available *in vivo* (14%) The riboflavin in this preparation was completely unavailable to two of the seven subjects who consumed it

Data on rate of excretion of riboflavin after ingestion of the eight preparations were compared with those for the combined 5 mg standards in Fig 1 A semilog plot was used, as suggested by Swintosky, *et al* (6) The peak urinary excretion rate declined with increasing disintegration time and was directly related to physiological availability The peak of riboflavin excretion occurred earlier with the standard than with any of the preparations studied The maximum rate of urinary excretion (0 40 mcg /min) found with the product of lowest physiological availability (product D) was only 4 2% of that found with the standard (9 45 mcg /min) The excretion curve found with product A was similar in shape to that found with the standard, except that the highest excretion rate recorded was only 5 92 mcg /min as compared to 9 45 mcg /min. for the standard Products B, C, and D showed low peak excretion rates and low physiological availability Product E, which was 51% available *in vivo*, showed a peak excretion rate of 5 15 mcg /min However, the rate of excretion found with this product declined somewhat more rapidly than did that of the standard The peak excretion rate attained with product F was only 5 48 mcg /min , but the riboflavin in this product was excreted more slowly than that in the standard dose, resulting in the highest availability (81%) of the products tested Products G and H had peak excretion rates of only 1 71 and 2 89 mcg /min , respectively

In further studies, the details of which are not reported herein, the physiological availability of riboflavin was determined in two pelleted "timed release" preparations containing amphetamine and vitamins and prepared by the same manufacturer The riboflavin in one preparation was fully available *in vivo*, whereas that in the second preparation was only 36% available *In vitro* disintegration times could not be determined on these pelleted products by the U S P procedure

In vitro disintegration times on four of the preparations studied herein were also determined by use of the fluted disks described in U S P XV Second Supplement (13) The results were plotted against percentage physiological availability in Fig 2 For comparison, the data of Chapman, *et al* (1), obtained with rubber disks, and those of the present studies obtained with solid plastic

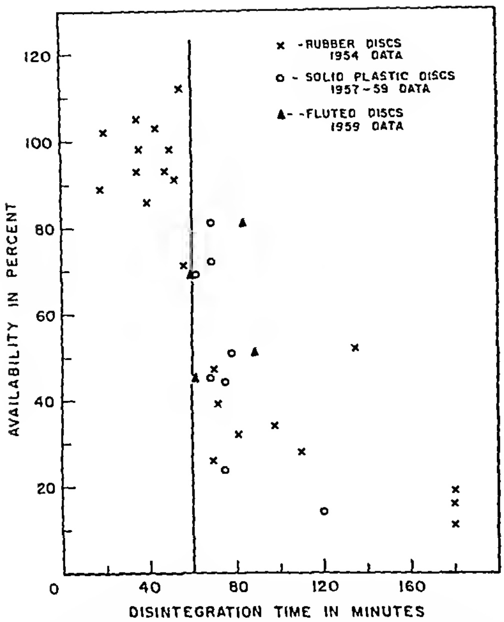

Fig 2 —Relation between *in vitro* disintegration time of sugar coated tablets and physiological availability of riboflavin.

disks, were also plotted The relationship found between *in vitro* disintegration time and *in vivo* availability was similar with all three types of disks, i e , of 33 different tablets tested, none with *in vitro* disintegration times exceeding sixty minutes were fully available *in vivo*.

DISCUSSION

The results of the present studies confirm those published previously (1-3) and would appear to remove any doubt there may have been concerning the validity of the sixty-minute time limit suggested for the *in vitro* disintegration of sugar coated tablets containing riboflavin A disintegration time of sixty minutes obtained by the procedure used herein would be approximately equivalent to ninety minutes by the U S P procedure. It has recently been proposed that the time limit for decavitamin tablets be reduced from four hours to two hours (15). The results of the present studies would indicate that the riboflavin in sugar coated tablets requiring two hours for *in vitro* disintegration by the U S P test would not be fully available to many subjects It is obvious that the one-hour time limit for tablets containing riboflavin only applies to those tablets which disintegrate uniformly It does not hold for tablets in which the riboflavin is on the outside of a relatively resistant core, or beside a more resistant layer. It is still possible of course, as Endicott and Kirchmeyer (4) have pointed out, that a somewhat different relationship might be obtained with other vitamins which are more soluble than riboflavin This point is largely academic, however, since most multivitamin tablets contain riboflavin and if a longer disintegration time were permitted, the riboflavin content of such tablets would not be available to the body

As was pointed out previously (16) the use of urinary excretion data to evaluate the release of drugs requires recognition of the fundamental relationship that exists between concentration or amount of drug in the blood and other fluids of distribution, on the one hand, and excretion rate of the drug on the other. It has been established both by experimental work and theoretical considerations that for several drugs, the excretion rate is directly proportional to the amount or concentration of drug or other exogenous substances in the blood This relationship has been shown to be true for creatinine, xylose, and penicillin. There is considerable evidence (17) that, in human subjects, the rates of urinary excretion of water-soluble vitamins are directly proportional to tissue levels and to the quantity of vitamins consumed, if the subjects are subsisting on an adequate diet. There seems little doubt, therefore, that measurement of the rate of urinary excretion of riboflavin, after dosing normal subjects with various riboflavin preparations, provides a valid measure of the availability of the dose to the body and of tissue levels produced by the dose. Thus, marked differences in blood levels of riboflavin might be expected after ingestion of the products tested in the present studies For instance, it seems reasonable to assume that the blood level of riboflavin found with product D (14% available *in vivo*) would at no time be more than approximately 5% of that found with the standard It is obvious that such a product would be unlikely to yield desired clinical effects

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A Rapid and Sensitive Method of Quantitative Determination of Apomorphine*

By P. N. KAUL, E. BROCHMANN-HANSSEN, and E. LEONG WAY

A rapid and specific assay procedure has been developed for the routine determination of apomorphine. The method involves oxidation of the compound by mercuric chloride and ultraviolet absorption analysis of the extracted reaction product at 330 mμ. The product obeys Beer's law over a wide range of concentration. Optimum conditions for the reaction have been investigated. The method has been applied to the recovery of added apomorphine from aqueous solutions and biological materials. As little as 0.1 mcg. per ml. can be assayed by the procedure.

APOMORPHINE exhibits unique pharmacological properties when administered to different animal species. Although used clinically as an expectorant and emetic, its usage for stimulating race horses has also been suspected. Detection of apomorphine, illegally administered to such animals, and studies on its metabolism require a sensitive and specific method for its identification and quantitation.

Although several more or less specific color reactions have been developed for qualitative detection of apomorphine (1, 2), no satisfactory method is available for its quantitative determination in microquantities. Titration procedures in aqueous or nonaqueous media (3, 4) are not sufficiently sensitive or specific for most biological work. General phenol reactions, adapted to quantitative determination of apomorphine (5), also lack specificity.

Apomorphine is a strong absorber of ultraviolet radiation, and it can, if of sufficient purity, be determined by ultraviolet spectrophotometry (6). However, because of its labile nature, it has proved difficult to purify this compound to the extent that a low blank value may be obtained from biological materials, without appreciable loss of apomorphine.

In 1915, Grimbert and Leclere (7) reported a color reaction for apomorphine based on its oxidation by mercuric chloride in a weakly basic medium of sodium acetate. The oxidation product was extracted with amyl alcohol which acquired a blue color. This reaction is used as a test for identity of apomorphine in the French Pharmacopeia (8), and is said to have a sensitivity of about 2 p. p. m.

In this paper, the original color reaction of Grimbert and Leclere has been studied, modified to further increase its sensitivity, and adapted to quantitative determination of apomorphine. The resulting method has been found to be of particular value for analysis of microquantities of the compound in biological materials because naturally occurring catecholamines, related structurally to apomorphine, do not interfere.

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EXPERIMENTAL

Reagents and Equipment.—Saturated mercuric chloride solution: 5 Gm. of mercuric chloride is dissolved in 100 ml. of water by heating on a steam bath, the solution is allowed to cool to room temperature, and filtered; isoamyl acetate, Mallinckrodt A R grade; McIlvaine buffer solution, pH 6.0 (9); apomorphine hydrochloride, U. S. P.; 50-ml. glass-stoppered centrifuge tubes; a suitable pH meter, Leeds Northrup model was used; and a suitable spectrophotometer for recording absorbance at 330 mμ. The Cary model 11 recording instrument was used for the work reported here.

Analytical Method.—The solution to be analyzed is diluted to a concentration of about 15 to 30 mcg. per ml. of apomorphine. To a 50-ml. glass-stoppered centrifuge tube are added 4 ml. of the diluted apomorphine solution, 1 ml. of the buffer solution, and 3 drops of the mercuric chloride solution. The mixture is heated in a water bath at 70° for three to four minutes, and then cooled to room temperature under running water. Ten milliliters of isoamyl acetate is added with a pipet and the tube shaken for one minute. The phases are separated by centrifugation, and the absorbance of the isoamyl acetate layer determined at 330 mμ against the solvent. The amount of apomorphine in the 4-ml. aliquot is obtained from a standard curve prepared from known solutions analyzed in the same way.

Spectral Characteristics.—Figure 1 shows the absorption spectrum of the colored reaction product in isoamyl acetate. The oxidation product exhibits two maxima at 540 mμ and 400 mμ in the visible region, and a sharp maximum of much greater absorptivity at 330 mμ. In amyl alcohol, the extraction solvent originally suggested by Grimbert and Leclere (7), the absorptivities are only about one-half of the corresponding values in isoamyl acetate. It is interesting to note that although the reaction is a colorimetric identification test for apomorphine, the absorbance measurement at 330 mμ in the ultraviolet region serves as a sensitive means for its quantitative estimation. The reagent blank at this wavelength reads zero absorbance.

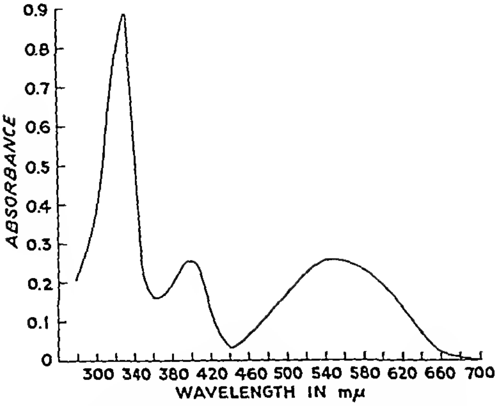

Fig. 1.—Absorption spectrum of the oxidation product of apomorphine in isoamyl acetate.

The oxidation product exhibits different colors in different solvents, perhaps due to the formation of different π-complexes. The color is green in water; blue in methanol, ethanol, amyl alcohol, and chloroform; pink in benzene, ether, and carbon tetrachloride; and violet in isoamyl acetate. In all these solvents, the peak at longer wavelength in the visible range shows a considerable shift, whereas there is only a slight shift in the ultraviolet peak.

Effect of pH.—The oxidation was carried out as described under "Analytical Method" at 70° for four minutes, while the pH was varied from 2.2 to 8.0 with McIlvaine buffers. The effect of pH on the reaction is illustrated in Fig. 2. Maximum absorbance values are obtained at pH 6.

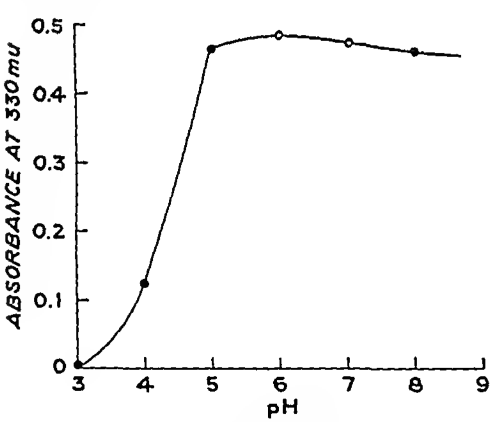

Fig. 2.—Effect of pH on the oxidation reaction of apomorphine.

Effect of Temperature and Time.—Figure 3 shows the temperature dependence of the reaction at a constant pH of 6.0 and a heating time of six minutes. The combined effect of time and temperature on the reaction is illustrated in Fig. 4.

At room temperature the reaction is slow, but the rate increases rapidly with an increase in temperature. Although various time-temperature combinations above 60° will give satisfactory results, a heating time of three to four minutes at 70° was chosen as most convenient. At lower temperatures, the reaction never reaches an optimal maximum. Prolonged heating results in decomposition of the reaction product, more so, however, at lower than at higher temperatures.

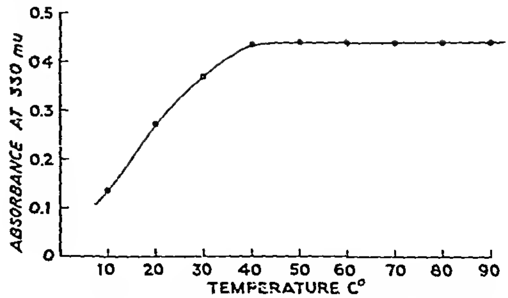

Fig. 3.—Effect of temperature on the oxidation reaction of apomorphine.

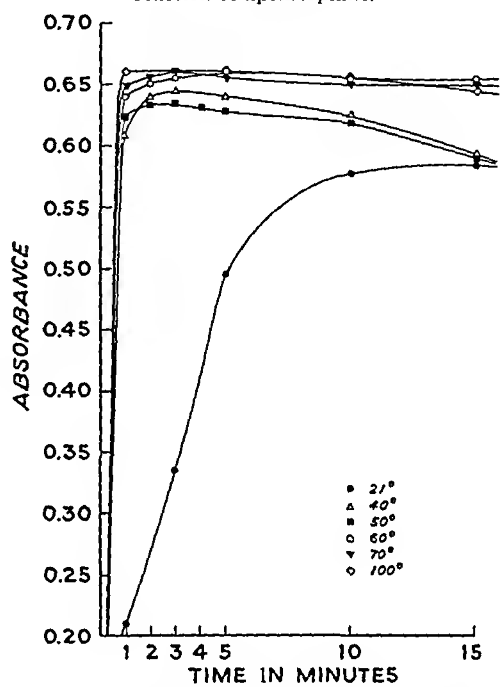

Fig. 4.—Effect of time of heating of the reaction mixture at different temperatures. Absorbance readings at 330 mμ.

Effect of Concentration.—The effect of concentration of apomorphine on the reaction is given in Fig. 5, which shows that the reaction obeys Beer's law over a wide range.

Specificity.—The color reaction was carried out with catechol, epinephrine, norepinephrine, and dihydroxyphenylalanine (DOPA). None of the colored reaction products given by these substances showed any absorbance at 330 mμ.

Procedure for Biological Materials.—While the analytical method described above is directly applicable to simple apomorphine preparations, such as injections or tablets, analysis of more complex preparations or biological fluids requires an extraction and purification step prior to the actual determination. The extraction procedure is: to a 4-ml. aliquot of diluted preparation in a separatory funnel are added enough 10% ammonia solution to give a pH of 6 to 7 and 20 ml. of C. P. ethyl acetate. The mixture is shaken for two minutes and the phases

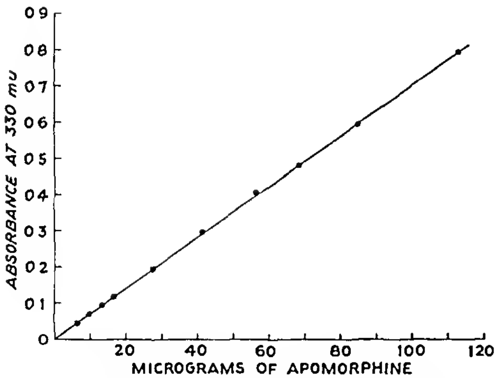

Fig. 5.—Standard curve for the oxidation reaction of apomorphine.

allowed to separate. The aqueous phase is drained into another separatory funnel and the organic phase washed with 1 ml. of water which is then combined with the aqueous phase. The ethyl acetate layer is shaken immediately with 5 ml. of 0.05 N hydrochloric acid and set aside while the combined aqueous layer and wash from the first extraction is shaken with 10 ml. of ethyl acetate. After separation of the two phases, the ethyl acetate layer is again washed with 1 ml. of water, the wash being combined with the aqueous layer and the ethyl acetate layer added to the ethyl acetate hydrochloric acid mixture. The extraction of the original aqueous phase and washing is repeated a third time with 5 ml. of ethyl acetate. The combined ethyl acetate extracts and the 0.05 N hydrochloric acid are shaken for about two minutes. The acid extract is collected in a 10 ml. volumetric flask, and the extraction repeated with 3 ml. and 2 ml. of 0.05 N hydrochloric acid. The final volume is adjusted to the mark with acid. The color reaction is carried out on a 4 ml. aliquot as described under "Analytical Methods," with the exception that the McIlvaine buffer used in these experiments should be pH 6.4 and five times the usual strength. This produces the desired pH of 6.0 in the reaction mixture.

Addition and Recovery Experiments.—Known amounts of apomorphine hydrochloride were dissolved in acidified water and urine specimens. Aliquots of 4 ml. were subjected to the extraction procedure described above, and finally assayed by the "Analytical Method." Table I gives the per cent recoveries from aqueous solution and urine specimens of humans, horses, and rabbits. It can be seen that while the recovery from urine was about 20% lower than that from aqueous solution, the respective recovery values from the urine of each species were quite consistent, varying less than ±3%. Zero absorbance at 330 mμ was observed when the blank specimens of all species were carried through the procedure.

DISCUSSION

Apomorphine is fairly stable in acidic aqueous medium, but its stability decreases rapidly with increasing pH. The addition of ascorbic acid has been used to stabilize apomorphine preparations (10). In our work the use of ascorbic acid to ensure greater recoveries of added apomorphine from biological fluids was of no value. Solvents also seem to have an effect on the stability of the free base. Chloroform favors a rapid decomposition, whereas ethyl acetate has been found to be the most satisfactory solvent for immiscible solvent extraction. It effects complete extraction of the base from an aqueous solution at pH 6 to 8, and if shaken with aqueous acid within three to five minutes, no detectable loss of apomorphine results.

The type of buffer used to maintain the optimum pH of the oxidation reaction mixture appears to be immaterial. Acetate and phosphate buffers of the same pH give similar results. The McIlvaine buffer was preferred because of its greater capacity in the desired range of pH ± 0.2.

Recoveries from urine were consistently lower than those from aqueous solutions. It appears that there is modification of some of the apomorphine either through complex formation with some substances normally found in the urine or because some of the drug is strongly adsorbed on the colloidal material present in it. Various attempts to recover the apparently modified or adsorbed fraction of the drug through (*a*) the hydrolysis of the residual urine left after extraction procedure and then re-extraction of the liberated apomorphine, if any, (*b*) the use of modified extraction procedure, involving excessive immiscible solvent extractions, and (*c*) modifying the conditions for the color reaction to improve the recovery, were all in vain. Further work along these lines to explain the discrepancy is in progress.

Relatively high specificity of the reaction for apomorphine and insignificant blank absorption values obtained from biological materials when carried through the procedure indicate the usefulness of the method for biological work. By increasing the path length of absorption and reducing the extraction volume of isoamyl acetate, the sensitivity

TABLE I.—RECOVERIES OF APOMORPHINE AFTER ADDITION TO AQUEOUS SOLUTIONS AND URINE

Material	Amount Added mcg.	Amount Recovered mcg.	Recovery, %
Aqueous Solutions	84.5	83.0	98.2
	84.5	83.8	99.1
	84.5	84.5	100.0
	84.5	82.8	98.1
	84.5	86.4	102.2
Human urine with added ascorbic acid	86.6	67.8	78.4
	86.6	69.8	80.6
	86.6	70.8	81.8
	86.6	67.8	78.4
Human urine without added ascorbic acid	85.5	72.0	84.5
	85.5	71.0	83.4
	85.5	72.2	84.6
Horse urine	20.9	16.2	77.5
	20.9	16.5	78.7
	20.9	16.5	78.7
	20.9	16.5	78.7
	20.9	16.5	78.7
	31.4	24.9	79.3
	31.4	25.3	80.6
	31.4	24.9	79.3
	31.4	26.0	82.8
Rabbit urine	95.5	78.8	82.5
	95.5	78.0	81.7
	95.5	79.5	83.3
	95.5	78.5	82.2

of the method may be increased many-fold As little as 0 1 mcg per ml of apomorphine can be easily estimated Such a sensitivity would find application in studying the *in vitro* enzymatic degradation of the drug Presently, the method is being used for studying the metabolism of apomorphine.

SUMMARY

The oxidation of apomorphine hydrochloride with mercuric chloride under standard conditions gives a green-colored product which turns violet when extracted into isoamyl acetate. The absorption peak at 330 mμ exhibited by the colored reaction product, in addition to its peaks in the visible region, has been used for quantitative determination of apomorphine Catecholamines and other related polyhydroxyphenols in urine do not interfere in the estimation.

Optimum conditions for the reaction have been determined By simple manipulations, as little as 0 1 mcg per ml can be determined A procedure has been described for the extraction of apomorphine from biological material and solutions containing interfering substances, and the color reaction has been applied to such extracts from human, horse, and rabbit urines.

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Kinetics of the Specific Base-Catalyzed Hydrolysis of Naphazoline*

By MARVIN J. STERN†, LOUIS D. KING, and ARNOLD D. MARCUS‡

The rate of the specific base-catalyzed hydrolysis of naphazoline has been found to be first order with respect to both naphazoline and hydroxyl ion, regardless of whether the naphazoline exists as the protonated or unprotonated species. A theoretical isothermal equation expressing the pseudo-first order specific rate constant as a function of the hydronium ion and hydroxyl ion activities has been derived, and at 25° appears to hold true over a ten million-fold variation in catalyst (hydroxyl ion) concentration. The activation energies and frequency factors for both the hydrolyses of protonated naphazoline and unprotonated naphazoline have been experimentally determined. The reaction involving protonated naphazoline is favored by frequency while the reaction involving unprotonated naphazoline is favored by energy. The frequency effect is the greater so that, at normal temperatures, protonated naphazoline hydrolyzes about one thousand times faster than the unprotonated form. Postulations for the mechanisms of the reactions have been made in light of the kinetic data and a generalized equation for calculating the observed rate constant has been devised.

ALTHOUGH the hydrolytic degradation of esters, amides, imides, and other carbonyl compounds is well recognized, the ability of other structures to degrade through a hydrolytic mechanism is often overlooked Such oversights may be particularly costly in pharmaceutical research. Excellent examples of such "other" species are the imidazolines It is, therefore, the purpose of the present communication to describe the kinetics of the specific base-catalyzed hydrolysis of a typical imidazoline—naphazoline

Naphazoline, 2-(1-naphthylmethyl)-2-imidazoline, has been shown to be relatively stable in acidic or neutral solutions but readily prone to hydrolysis in basic solutions The first step in this reaction (1) results in the formation of 1-naphthylacetylethylenediamine, which, upon more vigorous treatment (2), undergoes further cleavage to form 1-naphthylacetic acid and ethylenediamine. These reactions can be represented by the following scheme:

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This paper is based on a thesis presented to The Graduate School of Rutgers University by Marvin J Stern in partial fulfillment of the requirements for the degree of Master of Science

Portions of this paper are adapted from the honorable mention winning manuscript submitted by Marvin J Stern in the Eastern Region, 1958, Lunsford Richardson Pharmacy Award competition

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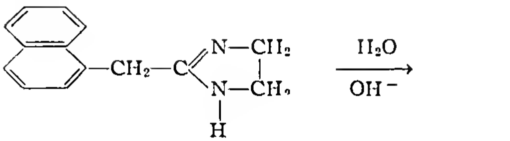
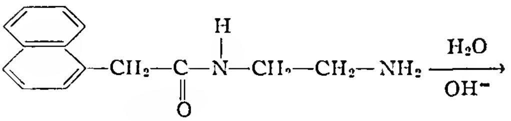
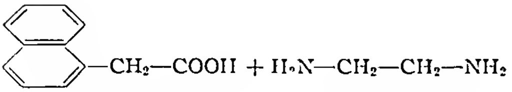


Naphazoline



1 Naphthylacetylethylenediamine



1 Naphthylacetic Acid Ethylenediamine

While both steps in the foregoing reaction scheme offer interesting subjects for kinetic studies, it is obvious that only the first step would be of concern in pharmaceutical formulation efforts, since this step is responsible for the disappearance of the intact physiologically active species from its solutions As a result, it was decided to examine the kinetics of this reaction in detail with the further objective of utilizing the kinetic data in proposing a reaction mechanism

THEORETICAL CONSIDERATIONS

Naphazoline, like all weak bases, exists in aqueous solutions in both protonated (salt) and unprotonated (base) forms As a result, any equation describing the rate of hydrolysis must include both potential substrates The rate expression would therefore be given by

$$-d[\text{Naph}]_T/dt = k_{\text{OH}^-\ S}\,[\text{OH}^-]^m[\text{Naph}]_S^n + k_{\text{OH}^-\ B}\,[\text{OH}^-]^{m'}[\text{Naph}]_B^{n'} \quad \text{(Eq 1)}$$

where the subscripts S, B, and T refer to the salt, base, and total, respectively, and the superscripts are the orders of the reactions with respect to the different species At constant hydroxyl ion activity, this becomes

$$-d[\text{Naph}]_T/dt = k_S'[\text{Naph}]_S^n + k_B'[\text{Naph}]_B^{n'} \quad \text{(Eq 2)}$$

where k_S' and k_B' are the pseudo nth and pseudo n'th order rate constants for the salt and base forms, respectively Dividing through by $[\text{Naph}]_T$ gives

$$-\frac{d[\text{Naph}]_T}{[\text{Naph}]_T} = \left(k_S' \frac{[\text{Naph}]_S^n}{[\text{Naph}]_T} + k_B' \frac{[\text{Naph}]_B^{n'}}{[\text{Naph}]_T}\right) dt \quad \text{(Eq 3)}$$

If it is assumed that n and n' are both equal to unity (i e , the reaction is first order with respect to either form of naphazoline), the terms $[\text{Naph}]_S/[\text{Naph}]_T$ and $[\text{Naph}]_B/[\text{Naph}]_T$ will be constant under conditions of constant pH Integration of Eq 3 will, therefore, give

$$\log\,[\text{Naph}]_T = -\frac{(k_S'[\text{Naph}]_S + k_B'[\text{Naph}]_B)t}{2.30\,[\text{Naph}]_T} + \frac{\log\,[\text{Naph}]_{T,o}}{2.30} \quad \text{(Eq 4)}$$

where $[\text{Naph}]_{T,o}$ is the initial total naphazoline concentration The overall reaction would, therefore, follow pseudo first order kinetics with the specific rate constant (k_{obs}') given by the equation

$$k_{obs}' = \frac{k_S'[\text{Naph}]_S + k_B'[\text{Naph}]_B}{[\text{Naph}]_T} \quad \text{(Eq 5)}$$

In other words, the observed specific pseudo first order rate constant is the "weighted average" of the individual salt and base constants

If the reaction involving the protonated form and the one involving the unprotonated form are of identical order with respect to hydroxyl ion, the relationship

$$k_{\text{OH}^-\ T} = \frac{k_{\text{OH}^-\ S}[\text{Naph}]_S + k_{\text{OH}^-\ B}[\text{Naph}]_B}{[\text{Naph}]_T} \quad \text{(Eq 6)}$$

can also be written In solutions of constant hydroxyl ion activity the expression relating the observed rate constant to the true rate constant is given by

$$k_{obs}' = k_{\text{OH}^-\ T}[\text{OH}^-]^m \quad \text{(Eq 7)}$$

Substitution of Eq 6 in Eq 7 gives, upon taking logarithms

$$\log k_{obs}' = -m\,p\text{OH} + \log \frac{k_{\text{OH}^-\ S}[\text{Naph}]_S + k_{\text{OH}^-\ B}[\text{Naph}]_B}{[\text{Naph}]_T} \quad \text{(Eq 8)}$$

The relative concentrations of the two forms of naphazoline can be determined by the buffer equation which in this case is

$$\log\,([\text{Naph}]_S/[\text{Naph}]_B) = pK_a - pH \quad \text{(Eq 9)}$$

where K_a is the dissociation constant of the protonated form, the conjugate acid of naphazoline base Solving Eq 9 for $[\text{Naph}]_S$ and $[\text{Naph}]_B$ in terms of $[\text{Naph}]_T$ gives

$$[\text{Naph}]_S = \frac{[\text{Naph}]_T[\text{H}^+]}{K_a + [\text{H}^+]} \quad \text{(Eq 10)}$$

and

$$[\text{Naph}]_B = \frac{[\text{Naph}]_T K_a}{K_a + [\text{H}^+]} \quad \text{(Eq 11)}$$

Substituting these values in Eq 8 gives

$$\log k_{obs}' = -m\,p\text{OH} + \log\left(\frac{k_{\text{OH}^-\ S}\,[\text{H}^+] + k_{\text{OH}^-\ B}\,K_a}{K_a + [\text{H}^+]}\right) \quad \text{(Eq 12)}$$

In solutions of low hydroxyl ion activity naphazoline exists almost entirely in the protonated form Equation 8 therefore becomes

$$\log k_{obs}' = -m\,p\text{OH} + \log k_{\text{OH}^-\ S} \quad \text{(Eq 13)}$$

Since $k_{\text{OH}^-,\ S}$ is constant at any particular temperature, if Eq 13 holds, a plot of log k_{obs}' *versus* pOH will be linear The slope of the line will be equal to the negative of the order of the reaction with respect to hydroxyl ion The "y" intercept of any such line will, of course, be equal to log $k_{\text{OH}^-,\ S}$

It will be shown in the following sections that the above equations appear to hold true and the assumptions made in their derivations are valid.

EXPERIMENTAL

Determination of Dissociation Constants.—The dissociation constant of naphazoline conjugate acid at each of three temperatures was determined by carefully preparing volumetric solutions of known concentrations of naphazoline hydrochloride and free naphazoline base.[1] This was accomplished by dissolving a known weight of the salt in recently boiled, double-distilled water and adding a known volume of a standard sodium hydroxide solution. Enough sodium chloride to bring the ionic strength to 0.6 was added and the solution was brought to volume with recently boiled, double-distilled water. The pH of the solution was immediately measured at the desired temperature using a Beckman Model H2 pH meter. The pKa value was calculated from Eq. 9.

The values of the dissociation constants so obtained were "semi-classical" values, i.e., based on hydrogen ion activities and all other values as concentrations. Since all of the measurements and calculations involved are actually in these units, the "semiclassical" dissociation constants are the proper values to use. Due to the presence of concentration terms, these constants had to be measured under conditions of the same ionic sterngth as the experimental naphazoline solutions in order to be meaningful.

Buffer Systems.—Buffer solutions were utilized in order to keep the hydroxyl ion activities constant during the kinetic runs. To obviate any error due to the effect of ionic strength on the reaction rates, all buffers were made to have ionic strengths of exactly 0.6 by adjustment with sodium chloride. The buffers were prepared by blending a solution of the base (or acid) at $\mu = 0.6$ and a solution of its salt at $\mu = 0.6$ until the desired pH was obtained. The solutions were made 0.2 M with respect to total buffer constitutents wherever the solubility permitted. The ionic strength figure of 0.6 was chosen since this represents the ionic strength of a 0.2 M solution of a 1:2 or 2:1 electrolyte.

Buffers were prepared to cover the pH range of approximately 6 to 14. The buffer types, in order of increasing pH, were: triethanolamine-triethanolamine hydrochloride, sodium borate-boric acid, sodium carbonate-sodium bicarbonate, diethylamine-diethylamine hydrochloride, and for very high pH's (above 12), dilute solutions of sodium hydroxide were used. The usual calcium or barium hydroxide could not be used due to the presence of sodium bicarbonate in the assay procedure (3). Phosphate salts also could not be used in buffers for this work since their presence seemed to prevent the colored complex developed in the assay from adhering to Beer's law, even in very dilute solutions.

The highest pH obtainable was 13.48 at 25°, corresponding to a 0.6 M sodium hydroxide solution. The fact that the buffer solutions were all made to have ionic strengths of 0.6 precluded the use of more concentrated hydroxide solutions.

[1] Due to the low solubility of the naphazoline base, the solutions were made on the order of 2.5×10^{-3} M with respect to the salt and base.

An important consideration in the use of buffer solutions is the fact that the hydroxyl ion activity changes with temperature by virtue of the thermal change of the value of the ion product of water. The change in hydroxyl ion activity is especially evident in the case of acid-salt buffers where the hydronium ion activity is kept relatively constant and changes in the ion product of water are reflected, for the most part, in changes in the activity of hydroxyl ion. Even in base-salt buffers, however, there is an appreciable thermal change in hydroxyl ion activity. The common practice of ignoring these changes was considered to be highly inaccurate, since calculations show that in a reaction first order with respect to hydroxyl ion, a change of as little as 0.2 pOH units represents an error of about 60% in the specific rate constant. This error is even greater in reactions of higher order with respect to hydroxyl ion.

In order to determine the correct values of the hydroxyl ion activities, the pH of each buffer was measured at the temperatures at which the kinetic runs were made. The pH's were subtracted from the thermodynamic pKw's at the temperatures in question (4) to give the pOH's.

Kinetic Runs.—One hundred milligrams of naphazoline hydrochloride was placed in a 100-ml. glass-stoppered volumetric flask, enough buffer solution added to make 100 ml., and the solution was filtered. The flask was placed in a constant temperature oil bath. After thermal equilibrium had been attained, a 5-ml. sample was removed from the flask, delivered into a glass vial, stoppered, and plunged into an ice-water mixture. In the case of high pH systems, where sudden cooling was not sufficient to quench the reaction, the vial contained a predetermined amount of hydrochloric acid sufficient to give a final pH of about 6. This sample was arbitrarily designated as the "zero-hour" sample. Additional samples were removed and quenched after appropriate time intervals. All samples were refrigerated until the time of assay.

Routine analyses for residual naphazoline were carried out by the colorimetric method of Slack and Mader (3). Prior to color development, the solutions were diluted with sufficient buffer so that the absorbance of the colored complex did not exceed 0.4. At higher absorbances, the buffer constituents appear to cause deviations from Beer's law.

The analytical procedure used to verify the stoichiometry of the reactions was the partition chromatographic procedure of Schwartz, Kuramoto, and Malspeis (2), as modified by Stern (5).

All volumetric withdrawals from the refrigerated samples were done with the vials at 0°.

RESULTS AND DISCUSSION

Dissociation Constants.—The pKa values of naphazoline conjugate acid were determined as described in the experimental section. Three solutions of different base:salt ratios were used for the determination of each pKa value and the average was taken. These "semiclassical" pKa values at 0.6 inonic strength are given in Table I. These values are in very good agreement with the results of Hall and Sprinkle (6) who found that the pKa's of the conjugate acids of bases of about the strength of

TABLE I —SEMICLASSICAL DISSOCIATION CONSTANTS OF NAPHAZOLINE CONJUGATE ACID AT μ = 0 6

Temperature °C	pKa
25 0	10 35 ± 0 02
35 0	10 13 ± 0 02
45 0	9 92 ± 0 03

naphazoline showed a linear decrease of about 0 02 for each degree increase in temperature

Verification of the Stoichiometry of the Reaction.—In order to show that hydrolysis was the only degradative pathway under the conditions employed in the kinetic runs, it was necessary to verify the stoichiometry of the reaction A kinetic run in pOH 4 55 borate buffer at 65° was chosen for this since this pOH and temperature were considered to be most representative of the conditions employed in the subsequent kinetic runs This buffer also corresponded in pH to the internal phase of the Celite 545 partition column employed in the assay (2, 5)

The samples were assayed for naphazoline, 1 naphthylacetylethylenediamine, and 1 naphthyl acetic acid The results are illustrated graphically in Fig 1 This plot clearly shows that hydrolysis is the only significant degradative pathway under the conditions employed since all of the naphazoline was accounted for and the total molar concentration of naphazoline plus its hydrolytic degradation products was constant The 1-naphthylacetylethylenediamine did not hydrolyze to any significant extent to form 1 naphthylacetic acid

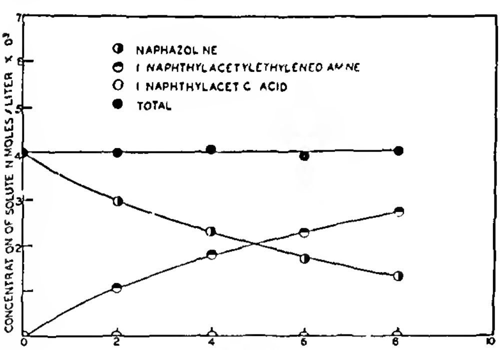


Fig 1 —Verification of the stoichiometry of the reactions

Effect of Naphazoline Concentration.—In Fig 2, the residual naphazoline concentrations from the kinetic run in pOH 4 55 buffer at 65° are plotted logarithmically against time From the straight line obtained, it appears that the rate of hydrolysis exhibits a first order dependency on the naphazoline concentration The fact that the same line was obtained when calculated from direct analysis of the residual naphazoline or from the analyzed concentrations of the decomposition products further illustrates the stoichiometry of the degradation All of the subsequent kinetic runs throughout the entire temperature and pH ranges that were investigated showed similar first order dependencies From the theoretical considerations presented, this would certainly indicate that the reaction is first order with respect to naphazoline regardless of whether the imidazoline exists as the protonated or unprotonated species

Effect of Hydroxyl Ion Activity.—In Fig 3 the logarithms of the pseudo first order rate constants have been plotted against pOH for the range where naphazoline exists almost exclusively in the protonated form Straight lines with slopes of −1 00 have been obtained, which, from Eq 13, indicates that the rate of hydrolysis of naphazoline is first order with respect to hydroxyl ion The plots have

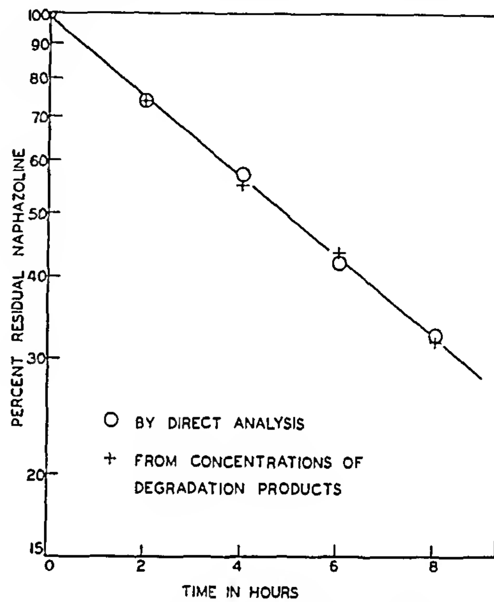


Fig 2 —Kinetic run at 65 0° in 0 1 M borate buffer, μ = 0 6, pOH = 4 55

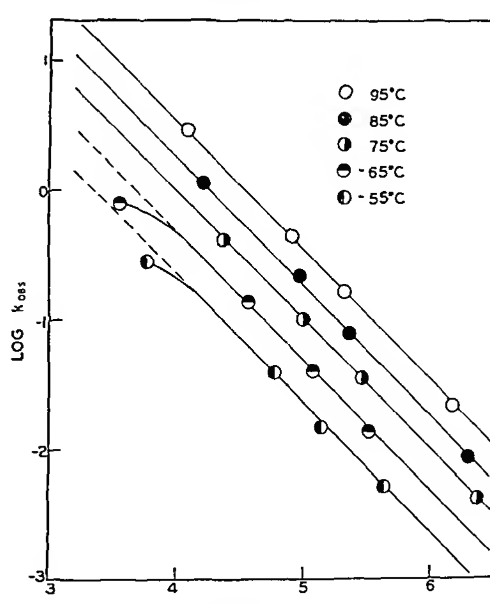


Fig 3 —Effect of hydroxyl ion activity on the rate of hydrolysis

been extrapolated to the "y" intercepts to obtain the logarithms of the second order specific rate constants which appear in Table II

It can be seen in Fig 3 that the points in the vicinity of pOH 3 5–3 8 do not fall on the lines, but below them These deviations are in accord with the theoretical considerations governing high pH (low pOH) systems where the presence of the unprotonated form of naphazoline is greatly increased All other things being equal, the unprotonated base would react at a slower rate than the protonated salt form due to the coulombic attraction between the latter and hydroxyl ions.

The effect of hydroxyl ion on the rate of hydrolysis at 25° in the pOH range 0–8 is given in Fig 4 While the points were experimentally determined, the line was not fitted to the experimental data, but instead, was drawn to represent the relationship expressed by Eq 12 The values of $k_{OH^-, S}$ and $k_{OH^-, B}$ used in solving Eq. 12 were 182 liters moles^{-1} hours^{-1} and 0 187 liters moles^{-1} hours^{-1}, respectively The value of $k_{OH^-, S}$ was obtained by extrapolation from results at higher temperatures The kinetic run at 25° at pH 12 98 was used for the determination of $k_{OH^-, B}$

Inasmuch as the derivation of Eq 12 required that the hydrolyses of both protonated and unprotonated naphazoline have the same kinetic dependency on hydroxyl ion, the excellent agreement shown in Fig 4 between the experimental results and theoretical equation indicates that this requirement was met Since it has been shown that the hydrolysis of protonated naphazoline is first order with respect to hydroxyl ion, the hydrolysis of unprotonated naphazoline must also be first order with respect to this ion.

TABLE II —SPECIFIC RATE CONSTANTS AT ELEVATED TEMPERATURES FOR THE HYDROLYSIS OF PROTONATED NAPHAZOLINE

Temperature °C	$k_{OH^-, S}$, liters moles^{-1} hours^{-1}
95	$3\ 4 \times 10^4$
85	$1\ 8 \times 10^4$
75	$9\ 8 \times 10^3$
65	$4\ 7 \times 10^3$
55	$2\ 2 \times 10^3$

The temperatures were controlled to ±0 05° or better

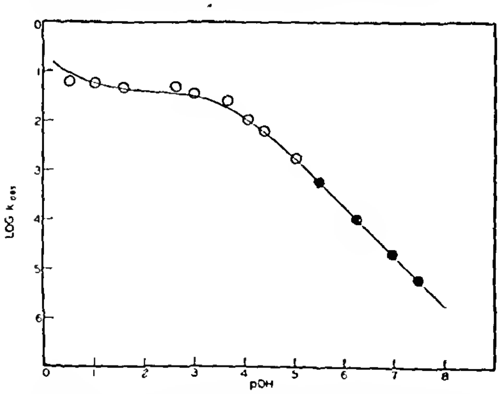


Fig 4 —Effect of hydroxyl ion activity on the rate of hydrolysis at 25 0°. The line represents the relationship expressed by Eq 12 The open circles are experimental points determined at 25 0° The solid circles are experimental points determined at other temperatures and extrapolated to 25 0°.

Temperature Dependencies of the Reactions.—The Arrhenius-type plots which appear in Figs 5 and 6 show the temperature dependencies of the reactions Difficulty was encountered during attempts to determine the effect of temperature on the rate of hydrolysis of the unprotonated form of naphazoline. The difficulty evolved from the fact that at normal temperatures the specific rate of hydrolysis of the protonated form is about one thousand times faster than that of the unpro-

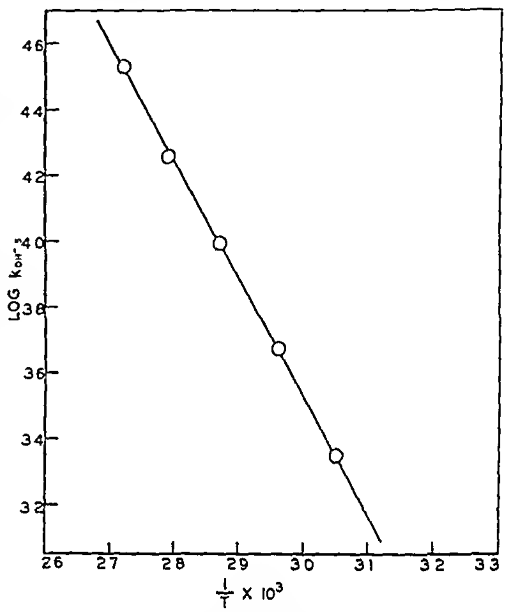


Fig 5 —Effect of temperature on the rate of hydrolysis of protonated naphazoline.

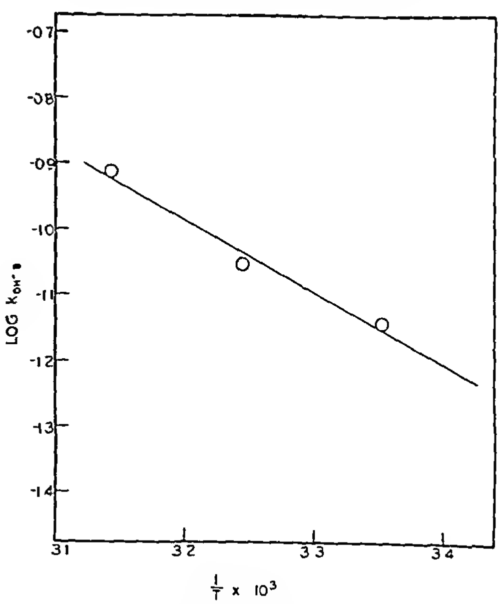


Fig 6.—Effect of temperature on the rate of hydrolysis of unprotonated naphazoline.

tonated form. Thus, even at pH 13.48, the highest pH that could be used, where the concentration of the unprotonated form is over thirteen hundred times greater than the concentration of the protonated form, the hydrolysis of the latter still accounts for over two-fifths of the overall degradation rate. Any rate constant for the hydrolysis of the unprotonated form derived under such conditions is, at best, an approximation, as would be any thermodynamic or kinetic value calculated from such rate constants. It was felt, however, that such approximations would still yield useful information. In Fig. 6, the regression line was drawn using the method of least squares (7).

The activation energy and frequency factor for the hydrolysis of protonated naphazoline have been calculated from the plot in Fig. 5. These were found to be 16.4 kilocalories per mole and 1.91×10^{14} liters moles^{-1} hours^{-1}, respectively. The values of the same parameters for the hydrolysis of unprotonated naphazoline, determined from Fig. 6, were found to be 4.9 kilocalories per mole and 3.0×10^{2} liters moles^{-1} hours^{-1}, respectively.

A generalized equation for the specific rate constant for the base-catalyzed hydrolysis, modeled after the Arrhenius equation, can be written as

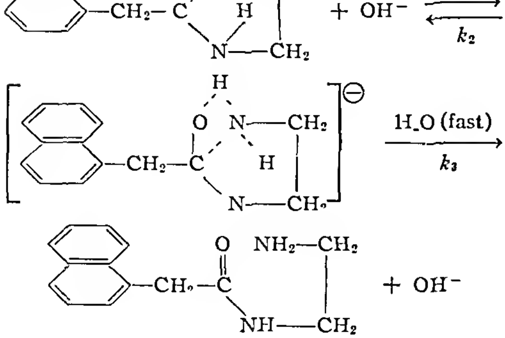
$$k_{obs}' = \frac{(1.91 \times 10^{14}\, e^{-16400/1.99T}\, [\text{Naph}]_S + 3.0 \times 10^{2}\, e^{-4900/1.99T}\, [\text{Naph}]_B)[\text{OH}^-]}{[\text{Naph}]_T} \quad \text{(Eq. 14)}$$

where T is the absolute temperature. Substituting the equivalents previously derived for $[\text{Naph}]_S$ and $[\text{Naph}]_B$ (see Eqs. 10 and 11) gives

$$k_{obs}' = \frac{1.91 \times 10^{14}\, e^{-16400/1.99T}\, K_w + 3.0 \times 10^{2}\, e^{-4900/1.99T}\, K_a[\text{OH}^-]}{K_a + [\text{H}^+]} \quad \text{(Eq. 15)}$$

Mechanisms of the Reactions.—While the kinetic data which have been presented are valuable in applications such as stability evaluation, they have much more fundamental utility. Kinetic data offer one important means of testing the validity of reaction mechanisms, and it is with the knowledge of mechanisms that the ultimate in predictions can be made. For this reason, mechanisms for the hydrolysis of naphazoline, which are in accord with the kinetic data, are proposed.

The hydrolysis of naphazoline base can follow the course represented by



While the exact nature of the intermediate cannot be unambiguously proved, the depicted structure represents a hybrid or compilation of the resonant forms that can be postulated. A model of the intermediate, constructed from Godfrey Molecular Models (8), indicates that the proposed structure is feasible.

The second step in the reaction, replacement of the hydroxyl group of the water, would be very fast. In other words, almost as soon as the unstable intermediate is formed, it reacts with the water. The value of k_3, therefore, would be much larger than that of either k_1 or k_2. Applying the steady state treatment to these reactions gives the following rate equation

$$-d[\text{Naph}]/dt = k_1\left(1 - \frac{k_2}{k_2 + k_3[\text{H}_2\text{O}]}\right)[\text{Naph}][\text{OH}^-] \quad \text{(Eq. 16)}$$

Since in dilute aqueous solutions, all of the terms within the parentheses are constant, they can be combined with k_1 to give an overall second order expression

$$-d[\text{Naph}]/dt = k_{\text{OH}^-}[\text{Naph}][\text{OH}^-] \quad \text{(Eq. 17)}$$

The value of k_{OH^-} should be very close to that of k_1 since the term $k_2/(k_2+k_3[H_2O])$ would be very small due to the large values of k_3 and $[H_2O]$.

According to Eq. 17, the reaction would be first order with respect to both naphazoline and hydroxyl ion. The experimental data appear to satisfy this requirement.

The fact that, in the proposed mechanism, hydroxyl ion is both a reactant and a product would make the reaction a true specific base catalyzed hydrolysis.

Although the depicted mechanism is for the hydrolysis of unprotonated naphazoline, a very similar scheme can be proposed for the hydrolysis of protonated napthazoline.

The Arrhenius frequency factor of 1.91×10^{14} liters moles^{-1} hours^{-1} for the reaction involving protonated naphazoline is a "normal" value for a bimolecular reaction in solution as calculated from collision theory (9). Upon cursory consideration, one might expect this parameter to be considerably greater by virtue of the fact that the reacting entities are of opposite electrical charge and, thus, exhibit coulombic attraction. This, however, is offset by two factors. First, in ionic interactions, the hydration shell has a restraining effect on the ions and will thus slow down the rate of collision. Second, the proposed mechanism of the reaction is such that the hydroxyl ion can attack from only one side of the naphazoline molecule, viz., the side in line with the double bonded imino nitrogen atom. For this reason, not every collision between ions of the proper energy will result in a reaction and the frequency factor will be lowered. The much lower

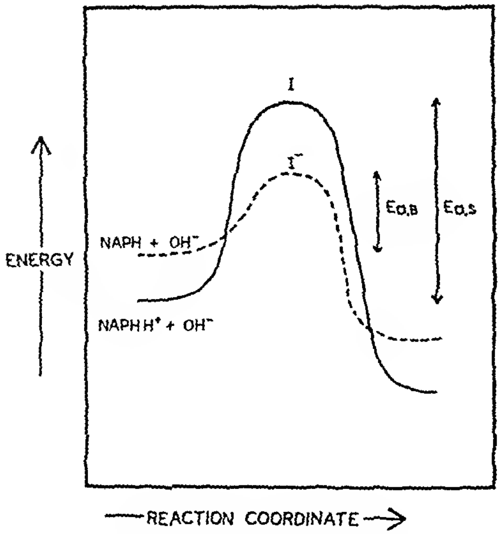


Fig 7.—Energetics of the reactions.

frequency factor ($3\ 0 \times 10^2$ liters moles^{-1} hours^{-1}) for the reaction involving unprotonated naphazoline is also to be expected since the frequency of collision for an ion and a neutral molecule would be much lower than for two oppositely charged ions

The relative activation energies can best be explained by the energy diagram in Fig. 7. Naphazoline conjugate acid, being charged, is more stable in solution than is the uncharged naphazoline base. Similarly, the activated complex formed in the reaction involving the free base has a charge and is more stable in solution than the uncharged intermediate in the reaction involving the conjugate acid (protonated) form. Since the activation energy is the difference in energy between the reactants and the activated complex, this parameter for the free base reaction would be considerably less than for the conjugate acid reaction. The experimental values of 4 9 kilocalories per mole and 16 4 kilocalories per mole, respectively, agree with the above considerations

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Analysis of Adrenergic Drug Action*

By R. S. McCUTCHEON† and RAYMOND P. AHLQUIST

Three analogs of arterenol were comparatively studied on several effector systems in dogs. The amines were: (*a*) ethylnorepinephrine (butanephrine), (*b*) isoproterenol (Isuprel), and (*c*) the N-isopropyl derivative of ethylnorepinephrine (Win-3046). These amines were compared to levarterenol and epinephrine as to potency and effect on arterial pressure, heart rate, intestinal motility, splenic capsule, and urine output. The effects on the splenic capsule best illustrates the general relationships found. Epinephrine, levarterenol, and ethylnorepinephrine produce primary splenic contraction; this effect is prevented by adrenergic blockade and is not modified by ganglionic blockade. Compounds (*b*) and (*c*), and (*a*) to some extent, induce a reflex splenic contraction by their depressor action. This effect is prevented by either adrenergic or ganglionic blockade. When administered simultaneously with epinephrine, (*b*) and (*c*) diminish the splenic contraction produced by the epinephrine.

THE ADRENERGIC RECEPTOR is the primary site of action of the adrenergic neurohormone and adrenergic drugs. It has been proposed that the adrenergic receptor occurs in two different forms (1). As far as smooth muscle is concerned, one of these receptors, known as the *alpha* receptor serves primarily excitatory responses The other receptor, known as the *beta* receptor, subserves primarily inhibitory responses Some adrenergic drugs activate both receptors Therefore, it would be desirable to have a convenient method for determining the differential potency on these two receptors. To investigate such a method, a comparative study in the anesthetized dog has been carried out using epinephrine, levarterenol, isoproterenol, ethylnorepinephrine, and the N-isopropyl derivative of ethylnorepinephrine (Win-3046).

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EXPERIMENTAL

Method.—Mongrel dogs of either sex weighing 10 to 15 Kg were anesthetized with pentobarbital sodium, 10 to 15 mg /Kg, administered intravenously thirty minutes after a subcutaneous injection of morphine sulfate, 10 mg /Kg The arterial pressure was recorded from either the femoral or carotid artery by means of a mercury manometer or a Statham transducer. The contraction of the

splenic capsule was recorded by a method previously described by Ahlquist, *et al.* (2). In brief, this method consists of exposing the spleen through a midline incision, fixing one edge of the spleen to a rigid plastic support, and connecting the opposite edge to an isotonic displacement gauge. The amount of splenic contraction is recorded as centimeters of galvanometer trace deflection on the photokymographic record.

A series of six experiments were made on the gut, urine flow, and spleen. The drugs were injected alternately in the following doses. Levarterenol, epinephrine, and isoproterenol were each given in doses of 0.01 ml./Kg of a 0.001 *M* solution. Win-3046 was injected in a dose of 0.02 ml./Kg. of a 0.001 *M* solution, and ethylnorepinephrine as 0.02 ml./Kg. of 0.01 *M* solution. These doses were, at times, doubled or halved in order to observe the relative responses better. In every case, in this and subsequent injections, decomposition of the amines was prevented by dissolving them in a solution of chlorobutanol and sodium bisulfite, 0.1% each. When blocking drugs were used they were injected in the doses shown below. The number of injections per experiment varied from 20 to 30. Control levels were obtained for each of the responses being measured and comparisons were made with the responses produced with the drugs under investigation, both before and after the use of the blocking agents. After each injection the response was allowed to return to control levels before the next injection. In these experiments the urine output from the renal pelvis was recorded with an electronic drop counter through a catheter inserted into the ureter. The activity of the small intestine of the animals in the same series was recorded by means of a small, water-filled balloon inserted into the lumen of the gut. The pressure developed by each intestinal contraction was recorded by means of a Statham transducer.

In another series of 15 experiments, dogs were given a constant infusion of one of the following drugs: ethylnorepinephrine, Win-3046, levarterenol, or isoproterenol and during this infusion each of the other drugs was injected intravenously every five minutes until each had been injected five to eight times. The infusions were administered by means of a drip bottle or by a syringe actuated by a stepping relay controlled through an electronic counting device (3). Ethylnorepinephrine was infused at a rate of 21.5 mcg./Kg./minute, Win-3046 was infused at a rate of 25.3 mcg./Kg./minute, levarterenol at a rate of 3.13 mcg./Kg./minute, and isoproterenol at a rate of 2.28 mcg./Kg./minute. In each case the infusions lasted one hundred to one hundred and twenty minutes. These doses were adjusted to give comparable results in each case. A few of the animals were given atropine, 0.5 mg./Kg., and when appropriate, mecamylamine (Inversine), 1 mg./Kg., was used as a ganglionic blocking agent or phenoxybenzamine (Dibenzyline) was used as an adrenergic blocking agent in a dose of 5 mg./Kg. All drugs were injected intravenously. A comparison was made of the results of the injections with the controls and with the results obtained after each type of block. During the infusions, blood pressure was recorded as indicated above and heart rates were recorded with an electrocardiograph and counted from the photokymographic tracing.

In comparative studies on adrenergic drugs we have found it convenient to use a "standard dose" of epinephrine as the basis for comparison. This standard dose is 0.01 ml./Kg. of a 0.001 *M* solution. This is roughly equivalent to about 2 mcg. of free base per Kg. The doses of the amines used in this study are expressed as fractions or multiples of this standard dose.

RESULTS AND DISCUSSION

The comparative effects of the amines on mean arterial blood pressure were found to be as described previously (1, 4). Levarterenol was the most potent, and epinephrine was somewhat less active. Ethylnorepinephrine produced a depressor response with the first dose and an increasing pressor response with subsequent doses (5). When given by infusion, ethylnorepinephrine produced a pressor response. Win-3046 and isoproterenol produced consistent depressor responses with the latter being the more active (6).

Following Dibenzyline, levarterenol produced only a small pressor response while all four of the other amines produced only depressor responses. These results indicate that levarterenol affects primarily the *alpha* receptor, epinephrine and ethylnorepinephrine affect both the *alpha* and *beta* receptors, while Win-3046 and isoproterenol affect only the *beta* receptor. When combinations of the drugs were injected, the results were not conclusive.

As has been previously described (1) acute changes in urine output roughly shows the relative renal vasoconstricting action of substances such as epinephrine. However, only with epinephrine and levarterenol was any consistent result found. With the other amines the urine output varied with the arterial pressure and no conclusions could be drawn regarding the relative action of these substances on the renal vascular bed.

The intestine of dogs not receiving atropine was relaxed by all of the amines except isoproterenol. This amine usually produced intestinal contraction. Following atropine, however, isoproterenol produced only relaxation. The relative potencies were as follows: isoproterenol was equal or more potent than epinephrine with the others less potent in this order: levarterenol, ethylnorepinephrine, and Win-3046. Mecamylamine did not affect these responses.

These results fit fairly well with the concept that the adrenergic inhibitor receptor of the gut is of the *alpha* type (2). The fact that isoproterenol is often more potent in this regard than epinephrine, however, is not consistent with this view. Recent studies have indicated that the canine ileum has both *alpha* and *beta* receptors, and that both receptors subserve relaxation (7, 8). We can conclude that the effect of these amines on the intact intestine is not a good criterion for relative receptor potency.

As previously reported (1), epinephrine was found to be the most potent substance in producing contractions of the splenic capsule. All of the other amines studied also produced splenic contraction when injected intravenously. The mechanism of action, however, was not the same for each. Figure 1 illustrates the control responses to epinephrine, isoproterenol, and Win-3046. As shown in Figs. 2 and 3, following the administration of a ganglionic blocking agent the splenic contraction produced by isoproterenol is abolished. The effect of Win-3046 is

TABLE I —AVERAGE SPLENIC CONTRACTION PRODUCED BY THE DRUGS SHOWN[a]

Dose	Epinephrine	Levarterenol	Butanephrine	Win-3046	Isoproterenol
S D[b]	5 91	1 92	(×10) 0 32	(×20) 2 37	0 81
S D / 2	3 83				
S D PM[c]	6 15	2 91	1 57		
S D PM / 2	4 22				

[a] Deflection on photokymographic record Contractions in centimeters, obtained from six animals with 18 to 30 injections per animal
[b] S D refers to standard dose
[c] PM is post mecamylamine (Inversine)

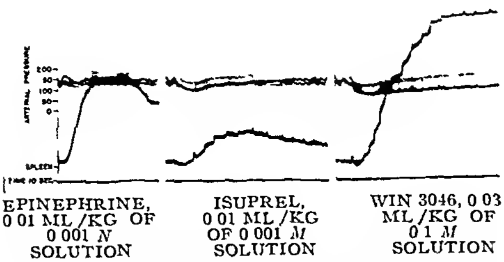

Fig 1 —Control responses of splenic contractions to epinephrine, isoproterenol, and Win-3046

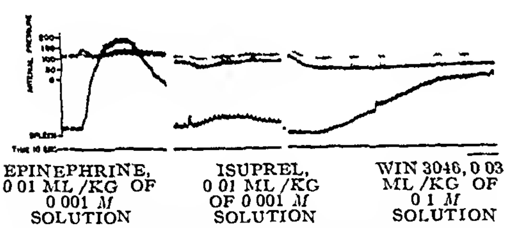

Fig 2 —Splenic contractions after administration of Inversine, 1 mg /Kg

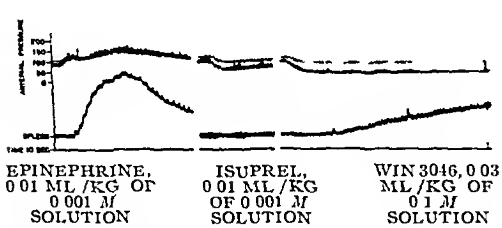

Fig 3 —Splenic contractions after second dose of Inversine, 1 mg /Kg

markedly reduced but this amine continues to produce some splenic contraction

Levarterenol and ethylnorepinephrine both produce splenic contraction These substances are potentiated by ganglionic blockade As described by Cameron (5) for the pressor response, ethylnorepinephrine becomes increasingly more potent in producing splenic contraction when repeated doses are given

These results indicate that the amines can produce contraction of the splenic capsule both by direct action and by reflex action due to the fall in blood pressure The latter effect is prevented by ganglionic blockade

Assuming then that splenic smooth muscle has only *alpha* receptors, results indicate that epinephrine is the most potent *alpha* activator, followed by levarterenol, ethylnorepinephrine, and Win-3046 Isoproterenol, having practically no action on *alpha* receptors, produces splenic contractions only through reflex action The results can be seen in Table I

However, some evidence has been obtained to show that the splenic capsule has some adrenergic *beta* receptors This is shown by administering epinephrine and Win-3046 simultaneously In every case the spleen contracted less when the mixture was given than when the same amount of epinephrine was given alone If both substances were producing splenic contraction by the same mechanism the effect should be additive and a greater contraction should result Since this is not the case one must assume that both epinephrine and Win-3046 activate some *beta* receptors in the spleen When the drugs are acting together the sum of this *beta* action is greater than the sum of the *alpha* effect of epinephrine and Win-3046 At the same time the epinephrine in the mixture prevents the depressor response to Win-3046 so that the reflex effect of the latter in causing contraction of the spleen is largely lost Therefore, the adrenergic inhibitory action on the spleen becomes more apparent

Table II shows the relative adrenergic receptor-activating potencies of the five catecholamines based primarily on their effects on splenic contraction

TABLE II —RELATIVE ADRENERGIC RECEPTOR-ACTIVATING POTENCIES

	alpha	beta
Epinephrine	+++++	++++
Levarterenol	++++	+
Ethylnorepinephrine	++	++
Win-3046	+	+++
Isoproterenol	+	+++++

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Activation Analysis of Certain Arsenic and Antimony-Containing Pharmaceuticals and the Effect of Beta-Emitting Isotopes*

By DAVID G. KAISER† and JOHN E. CHRISTIAN

Slow neutron activation analysis has been applied successfully to the analysis of pure samples of arsenic trioxide, carbarsone, oxophenarsine, antimony potassium tartrate, and stibophen, the average error being between 4 and 5 per cent. Sample processing and chemical separations were not required in the procedures. The effect of beta-emitting isotopes on the gamma-ray scintillation spectrometer was investigated and the detection levels of phosphorus-32 determined with and without shielding.

Perhaps the earliest examples of neutron activation analysis were reported by Hevesy and and Levi (1, 2, 3) who applied the procedure to the investigation of rare earth elements. The determination of arsenic in germanium dioxide was described by Smales and Brown (4) while Griffon and Barband (5) studied the distribution of arsenic in hair. Smales and Pate (6, 7, 8) published a series of papers on the determination of submicrogram quantities of arsenic. Hudgens and Cali (9) utilized the method to analyze for antimony in zirconium oxide. An analysis for arsenic in silicon was described by James and Richards (10). Recently, Smales, Mapper, Wood, and Salmon (11) determined trace quantities of arsenic, antimony, and copper in pure silicon.

The work reported here consisted of investigating the slow neutron activation analysis of arsenic and antimony-containing pharmaceuticals.

EXPERIMENTAL

Analysis of Arsenic and Antimony-Containing Pharmaceuticals.—For these experiments, polyethylene tubing (2 inches long $\times$ $^3/_{32}$ inch internal diameter $\times$ $^1/_4$ inch external diameter) was utilized as a sample container. The individual pharmaceuticals were dried according to the U. S. P. XV or N. F. X directions, and a sample placed in a 2-inch length of the tubing. The heat-sealed polyethylene tubes were then placed in an aluminum spacer which was subsequently inserted into a standard ANL[1] aluminum irradiatio ncontainer (6 inches high $\times$ $1^1/_4$ inches diameter). Samples prepared in this manner were subjected to $\sim 1 \times 10^6$ neutrons per square cm. per second for twenty-four to forty-eight hours. The induced radioactivity was determined with a RIDL[2] scintillation spectrometer consisting of a model 115 single channel pulse height analyzer, model 200T scaler, model 43A scintillation counter with a thallium-activated sodium iodide well-type crystal ($1^3/_4$ inch diameter $\times$ 2 inch thick with a well 0.650 inch diameter $\times$ $1^1/_2$ inch deep), and a model L-9 lead-iron shield. In later studies, a model 39-1 electronic sweep and count rate meter together with a Leeds and Northrup[3] Speedomax type G recorder were installed to provide an automatic recording instrument. The instrument was calibrated with cesium-137 and cobalt-60[4] to provide a maximum channel level of 1 Mev.[5] and a maximum channel width of 0.1 Mev. at a gain setting of $^1/_4$. A gain of $^1/_8$ doubled the channel level and channel width. A linearity study showed the instrument to be standardized with respect to peak energy *versus* channel level readings.

The investigated compounds and the selected solvents were as follows: arsenic trioxide and sodium hydroxide; carbarsone[6] and sodium hydroxide; oxophenarsine hydrochloride[7] and distilled water; antimony potassium tartrate and aqua regia; stibophen[8] and distilled water.

After irradiation, the polyethylene tubes were opened with a razor blade and the contents were transferred to tared, volumetric flasks. These samples were brought up to volume with the selected solvent and allowed to equilibrate thermally before pipetting an aliquot for analysis. One 500-λ portion was transferred into a 2 inch test tube together with two 500-λ rinsings and the test tube was stoppered with a paraffin-coated cork. In later studies, Neutraglas[9] ampuls of 5-ml. capacity were chosen for counting larger samples in the well-type crystal. The qualitative spectrum was determined by placing each sample in the counting well of the previously standardized gamma-ray scintillation spectrometer. Differential spectral determinations were performed at gains $^1/_4$ and $^1/_8$ with a channel width of "1." In each instance the spectrum compared with literature values and had the same energy distributions as standard reactor irradiated units.[10] Quantitative studies were delayed for twenty-four to forty-eight

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[1] ANL, Argonne National Laboratory.
[2] Radiation Iostrument Development Laboratory, Cbicago, Ill.

[3] Leeds and Northrup Co., Philadelphia, Pa.
[4] Cesium-137 and Cobalt-60 were obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.
[5] Mev.—million electron volts.
[6] Supplied through tbe courtesy of Eli Lilly and Co., Indianapolis, Ind.
[7] Mapharsen, supplied through courtesy of Parke Davis and Co., Detroit, Mich.
[8] Fuadin, supplied through courtesy of Winthrop Laboratories, New York, N. Y.
[9] Neutraglas ampuls, product of Kimble Glass Co., Toledo, Ohio.
[10] Standard reactor irradiated units of arsenic-76 and antimony-122 were obtained from Oak Ridge National Laboratory, Oak Ridge, Teon.

TABLE I.—RESULTS OF THE SLOW NEUTRON ACTIVATION ANALYSIS OF ARSENIC AND ANTIMONY-CONTAINING PHARMACEUTICALS[a]

Compound Investigated	Sample Weight, Gm.	Total Activity, Counts/min.	Calculated Activity of 50-mg. Sample[b] Counts/min.	Per Cent Error[c]
Arsenic trioxide[d]	0.1134	12,469	5,498	4.9
	0.2755	24,591	4,663	
	0.5405	49,120	4,544	
	0.5444	48,495	4,454	
Carbarsone	0.0530	61,304	57,835	0.3
	0.0819	94,169	57,487	
	0.1280	148,060	57,835	
	0.1512	176,887	58,492	
Oxophenarsine	0.1185	198,398	83,712	1.2
	0.1520	269,774	88,741	
	0.2444	427,564	87,472	
	0.2524	434,352	86,045	
Antimony potassium tartrate[e]	0.1396	4,618,418	1,654,161	6.1
	0.2325	9,436,490	2,029,352	
	0.3029	11,675,860	1,927,345	
	0.3742	11,701,100	1,563,482	
Stibophen	0.0557	107,651	96,808	5.8
	0.1034	210,884	127,345	
	0.1667	388,255	116,453	
	0.1864	459,721	123,315	

[a] Irradiations were conducted in the Argonne National Laboratory CP-5 reactor at a neutron flux of $\sim 10^6$. Exposures were of twenty-four to forty-eight hours' duration.
[b] The calculated activity was based upon a 50-mg. sample for comparison purposes.
[c] The per cent error was calculated from the following formulas:

$$R.\ D. \times 100 = \text{Per cent error}$$
$$R.\ D. = \frac{Md}{N}$$
$$Md = \sqrt{M_1^2 + M_2^2}$$
$$M = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

where: $R.\ D.$ = relative deviation, Md = mean error of difference, N = number of counts per unit time, M_1 = standard deviation of mean for sample, M_2 = standard deviation of mean for background, M = standard deviation of mean, Σd^2 = sum of the squares of the deviations from the arithmetic mean, and n = number of observations.
[d] The values reported are the first set obtained. Sixteen determinations were performed to establish reproducibility and yielded an average error of 5.7%.
[e] These samples received an exposure of one hour in a neutron flux of $\sim 10^{12}$, in addition to forty-seven hours at $\sim 10^6$.

hours and conducted by counting all of the samples at the same energy level. The channel width was increased to "5" and the channel level set to the peak maximum of the gamma radiation (0.558 Mev. for arsenic-76 and 0.566 Mev. for antimony-122). Each sample was subjected to 10 five-minute counting sessions, background subtracted, and the count corrected for decay. In this manner, qualitative and quantitative studies were made on arsenic trioxide, carbarsone, oxophenarsine hydrochloride, antimony potassium tartrate, and stibophen. The results are compiled in Table I.

Investigation of the Effect of Beta-Emitting Isotopes on the Gamma-Ray Scintillation Spectrometer.—Preliminary investigations showed that beta particles, produced during irradiation of chemical samples, caused an increase in detectable activity. This activity interfered with gamma-ray counting at the 0.558 Mev. arsenic-76 radiation peak, when submitted to a scintillation spectrometer examination. The energy distribution of phosphorus-32,[11] a pure beta emitter, is presented in Fig. 1. In the 0.500 to 0.600 Mev. region, the activity ranged from 3,600 to 2,800 counts per minute. In the lower energy regions, the activity increased to 35,000 counts per minute. A shielding experiment was designed to determine the possibility of eliminating this interfering radiation. The investigation was performed beneath the cesium-137 radiation peak, utilizing a channel width of "1" and a gain of $^1/_4$. The results are presented in Table II.

[11] Henceforth to be designated as P-32.

TABLE II.—RESULTS OF β-SHIELDING STUDY CONDUCTED WITH THE GAMMA-RAY SCINTILLATION SPECTROMETER

	Activity, counts/min.			
Type of Shield[a]	Background[b]	Cesium-137[c]	Phosphorus-32[d] 200 μc[e]	Cesium-137/Phosphorus-32 Activity Ratio[f]
None	3	27,590	13,624	2.0
Lucite[g]	1	25,508	1,120	22.7
Aluminum	3	23,237	1,067	21.8
Steel	1	19,971	933	21 4
Lead	1	15,157	711	21.3

[a] All shields were constructed according to the same specifications ($^3/_8$ inch internal diameter $\times$ $^5/_8$ inch external diameter) and had a minimum thickness of 3 mm.
[b] Background was determined with a 2-inch test tube containing 1.5 ml. of distilled water.
[c] This sample served as a calibration standard for the gamma-ray scintillation spectrometer. It emits two beta particles of 0 51 and 1.2 Mev. energies. The 2 6 minute half-life barium-137, a radioactive daughter, emits a 0 661 Mev. γ ray.
[d] Phosphorus-32 is a pure beta emitter of 1.71 Mev. energy.
[e] μc.—microcurie.
[f] This value was obtained by dividing the activity of cesium-137 by the activity of phosphorus-32 as determined under the conditions of this experiment.
[g] Lucite—E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

Comparing the results of the lead shielding with those without shielding, a reduction in the cesium-137 radiation by one-third is noted. The P-32 activity was reduced by a factor greater than 10 when the lead shielding was applied. Background deter-

TABLE III —THE DETECTION OF PHOSPHORUS-32 BETA PARTICLES AND BREMSSTRAHLUNG BY THE GAMMA-RAY SCINTILLATION SPECTROMETER

	Phosphorus 32 Activity, μc Activity Detected, Counts/min [a]												
	300	100	50	33 3	16 6	11 1	5 5	3 7	1 8	1 2	0 6	0 4	0 2
Unshielded[b]	22,394	7,998	3,627	3,968	1,131	877	481	262	121	114	51	24	15
Shielded[c]	2,215	451	222	144	56	37	22	12	6	4	4	3	2
Channel width 1, gain 1/4, 0 558 Mev arsenic-76 radiation peak													

[a] Uncorrected for background Each value is the average of 10 ten-minute counting sessions
[b] Contained in two inch test tubes
[c] Contained in two inch test tubes shielded by 3 mm of lead
Average background without shield, 5 counts per minute (average of 12 one hour counting sessions)
Average background with shield, 2 counts per minute (average of 12 one hour counting sessions)

minations were essentially the same with all of the shields

The P-32 activity, observed in conjunction with the various shields, was attributed to Bremsstrahlung, since calculations indicate that 785 mg /cm ^{2} of aluminum absorber will stop the 1 71 Mev beta radiation Three millimeters of lead corresponds to 3,860 mg /cm ^{2}.

Further work was conducted with the automatic recording attachment to study the effect of Bremsstrahlung resulting from P-32 activity Quantitative results (Table III) were determined by counting under the 0 558 Mev arsenic-76 radiation peak Activity resulting from P-32 was reduced to background at the $\sim$ 0 2 to $\sim$ 0 4 μc level, using the lead shield At the $\sim$ 0 2 μc level, without the lead shield, the P-32 activity was three times greater than the average background activity These experiments show the level of detection of P-32 Bremsstrahlung by the gamma-ray scintillation spectrometer and prove the fact that the presence of beta particles at microcurie levels will interfere with quantitative gamma counting.

SUMMARY AND CONCLUSIONS

1 This investigation was conducted to show the applicability of slow neutron activation to the analysis of pharmaceuticals Irradiations were performed at a neutron flux of $\sim 10^6$, in the case of pure compounds, to emphasize the possibilities of laboratory sources for routine analyses

2 The gamma-ray scintillation spectrometer, utilized in this investigation, was found to be sensitive to microcurie amounts of beta-emitting isotopes. Three millimeters Lucite, aluminum, steel, and lead shields were constructed to reduce the beta interference, but they were ineffective in completely controlling the Bremsstrahlung

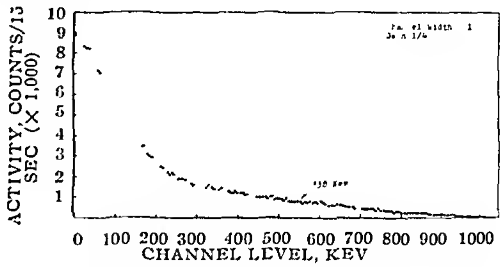

Fig 1 —Phosphorus-32 (50 μc.) energy distribution

3. The method developed was applied to the analysis of pure samples of arsenic trioxide, carbarsone, oxophenarsine, antimony potassium tartrate, and stibophen. Within the milligram level, the average error was between 4 and 5 per cent A higher neutron flux and/or a longer irradiation time would result in sensitivities at the microgram and submicrogram levels The primary advantage of the procedure is the minimum amount of chemical manipulation required..

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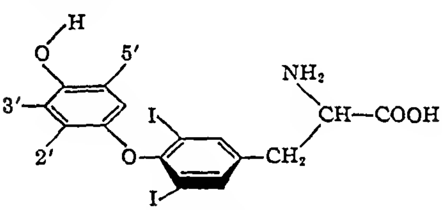
Thyroxine Analogs II*

Synthesis of 3,5-Diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine and Its 4′-Hydroxy Congener

By EUGENE C. JORGENSEN and PUSHKAR N. KAUL

The synthesis of thyroxine analogs, 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine and 3,5-diiodo-2′,3′-dimethyl-DL-thyronine is described. These compounds were prepared in order to study the effect on biological response of alkyl substituents in sterically oriented analogs of thyroxine. Both compounds displayed thyromimetic activity.

SINCE THE ORIGINAL SYNTHESIS of thyroxine by Harington and Barger (1), many analogs have been prepared in attempts to establish structural requirements for thyroxine-like activity. Recently (2), attention has been directed to a potential steric factor whereby the 3′ and 5′-positions of 3,5-diiodothyronine and related diphenyl ethers become nonequivalent in their spatial orientation following introduction of a 2′-alkyl substituent (I).



(I)

The 2′-substituent assumes a preferred orientation distal to the alanine-bearing ring, thus providing a minimal interaction between the 2′-substituent and the bulky 3,5-iodines and the ring bearing them Substituent groups on the prime ring may be located spatially by reference to their positions relative to the orienting 2′-alkyl group, and thus to the diiodophenylalanine ring.

Zenker and Jorgensen (2) have reported the synthesis of a series of 3,5-diiodo-4-(2′-alkylphenoxy)-DL-phenylalanines bearing additional 4′-hydroxyl and 5′-methyl substituents. These compounds reflect the orientation of a 5′-alkyl group in a position proximal to the diiodophenylalanine ring. Analogous compounds carrying 4′-hydroxyl and 3′,5′-dimethyl substituents have been shown to possess thyroxine-like activity (3, 4). For this reason, it was desirable to extend the series to provide a 3′-methyl group, oriented distal to the diiodophenylalanine ring as is the case for 3,5-diiodo-2′,3′-dimethyl thyronine (VI). Since the 5′-position was available for substitution, it was considered desirable to prepare the triiodo analog of this series, 3,5,5′-triiodo-2′,3′-dimethyl-DL-thyronine (VII). Analogs lacking the 4′-hydroxyl group have been reported (5) to act as thyroxine antagonists. The related compound, 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine (V), was therefore prepared to provide a distally oriented 3′-methyl group in this series of potential antagonists.

The synthetic route used was that developed by Hems and co-workers (6). 3,5-Dinitro-4-(*p*-toluenesulfonyloxy) - DL - phenylalanine - N - acetyl ethyl ester (II) was allowed to condense in the presence of pyridine with 2,3-dimethylphenol, yielding 3,5-dinitro-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (IIIa) or with 2,3-dimethyl-4-methoxyphenol yielding 3,5-dinitro - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (IIIb).

These dinitrodiphenol esters were hydrogenated to the diamines with Pd-on-charcoal catalyst which, without isolation, were tetrazotized and converted to the corresponding 3,5-diiodo analogs Hydrolysis with hydrochloric and acetic acids converted 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (IVa) to 3,5-diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine (V). To effect cleavage of the methoxyl group in 3,5-diiodo-4-(2′,3′-dimethyl-4′ - methoxyphenoxy) - DL - phenylalanine - N - acetyl ethyl easter (IVb), the compound was treated with hydriodic acid in acetic acid, yielding the desired 3,5-diiodo-2′,3′-dimethyl thyronine (VI). Attempts to form 3,5,5′-triiodo-2′,3′-dimethyl-DL-thyronine (VII) by the iodination of VI with iodine and potassium iodide in aqueous ethylamine were unsuccessful.

BIOLOGICAL RESULTS

3,5 - Diiodo -2′,3′ - dimethyl - DL - thyronine (VI) demonstrated thyroxine-like activity in the :

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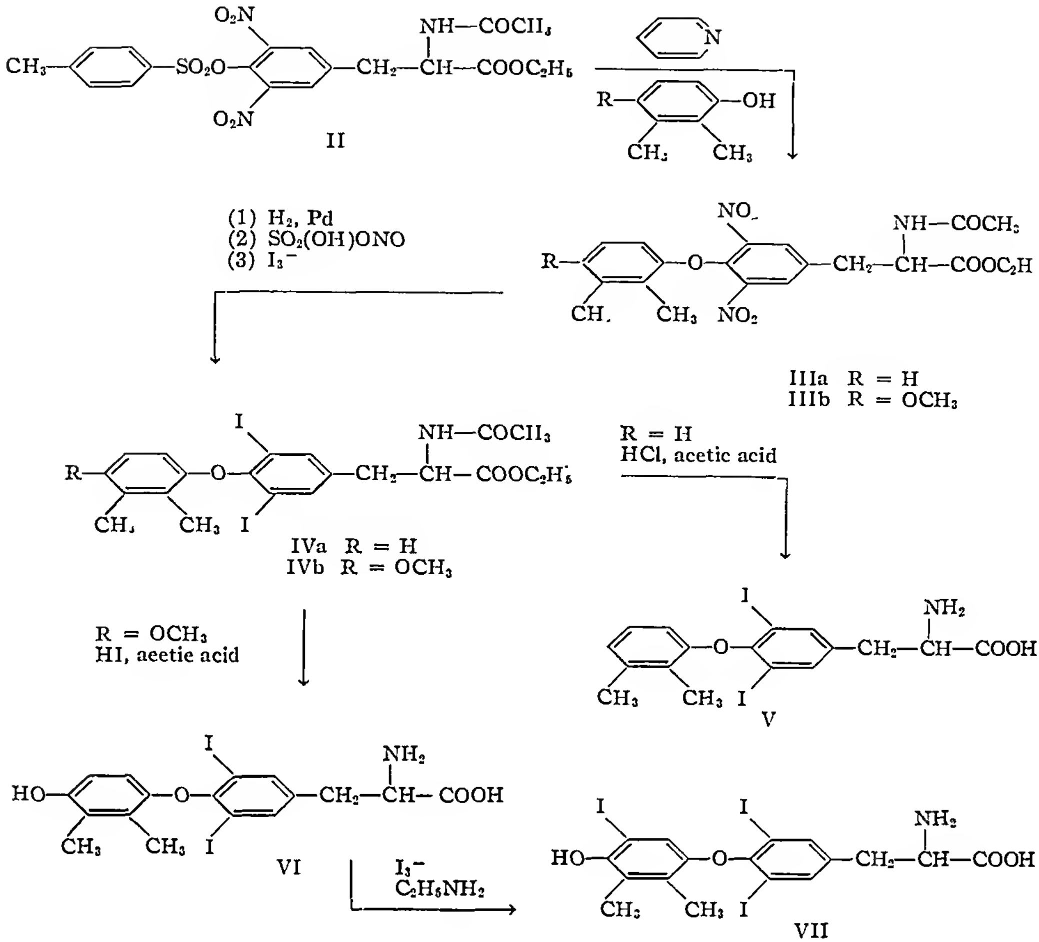
Previous paper in this series, *J. Am Chem Soc*, in press.



at 3 mg./Kg./day for ten days in the antigoiter assay of Cortell (7) and at a single dose of 20 mg./Kg. in the oxygen consumption assay of Holtkamp and Heming (8). 3,5-Diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine (V) was active at 7 mg./Kg./day for ten days in the antigoiter assay, but inactive in the test of oxygen consumption at a single dose of 20 mg./Kg. This represents the first report of a thyromimetic thyroxine analog unsubstituted in the 4′-position. A detailed discussion of these results relating stereochemical orientation of substituents to biological response will be submitted elsewhere.

EXPERIMENTAL[1]

3,5 - Dinitro - 4 - (*p* - toluenesulfonyloxy) - DL-phenylalanine-N-acetyl Ethyl Ester (II).—Crystallized from aqueous acetone, m. p. 160–162°, prepared in 75% yield from 3,5-dinitro-DL-tyrosine-N-acetyl ethyl ester[2] by the method of Barnes, *et al.* (5), who reported m. p. 157–158°.

2,3-Dimethyl-1,4-hydroquinone.—Prepared from 2,3-dimethylphenol[3] by the method of Smith and Tess (9) in 36% yield. Crystallized from benzene and sublimed at 130° (17 mm.), m. p. 219–220° (decompn.). Lit. (9), 223–224° (decompn.).

Anal.—Calcd. for $C_8H_{10}O_2$ (138.2): C, 69.55; H, 7.30. Found: C, 69.63; H, 7.16.

2,3-Dimethyl-4-methoxyphenol.—To a well-stirred solution of 2,3-dimethyl-1,4-hydroquinone (62.3 Gm., 0.45 mole) in sodium hydroxide (44.8 Gm., 1.12 moles) and water (350 ml.) at 10°, dimethylsulfate (68 Gm., 0.54 mole) was added in small portions over a period of two hours, and stirring continued an additional hour at 10° (10). The mixture was extracted with five 100-ml. portions of ether (Extract I), the aqueous solution was acidified with 6 *N* hydrochloric acid and extracted with four 100-ml. portions of ether (Extract II). Following removal of ether, Extract I yielded 34.0 Gm. of 2,3-dimethyl-1,4-dimethoxybenzene, m. p. 78–79°. Lit. (11), 78°. The residue following ether evaporation of Extract II was extracted repeatedly with boiling ligroin (30–60°). The combined ligroin extracts were evaporated and the residue crystallized from water, yielding 12.0 Gm. (18%) of 2,3-dimethyl-4-methoxyphenol, m. p. 95–96°. Lit. (9), 95–97°.

3,5 - Dinitro - 4 - (2′,3′ - dimethylphenoxy) - DL-phenylalanine-N-acetyl Ethyl Ester (IIIa).—3,5-Dinitro - 4 - (*p*-toluenesulfonyloxy) - DL - phenylala-

[1] Melting points obtained on Fisher-Johns melting point apparatus and are uncorrected. Microanalysis by the Microanalytical Laboratory, Department of Chemistry, University of California.
[2] Prepared by Mr. Robert Day.
[3] Aldrich Chemical Co., Milwaukee, Wis.

nine-N-acetyl ethyl ester (16.5 Gm., 0.033 mole) was dissolved in dry chloroform (100 ml.) and heated under reflux with dry pyridine (20 ml., 0.25 mole) for thirty minutes. 2,3-Dimethylphenol[4] (8.1 Gm., 0.066 mole) was added and the mixture heated at reflux temperature for an additional three hours. After cooling, the chloroform solution was washed successively with 2 *N* hydrochloric acid, water, 2 *N* sodium hydroxide, and water, dried over calcium chloride, filtered, and the chloroform removed under reduced pressure. The residue was crystallized from ethanol, yielding 3.6 Gm. (25%), m. p. 134–135°.

Anal.—Calcd. for $C_{21}H_{23}N_3O_8$ (445.4): C, 56.62; H, 5.21. Found: C, 56.44; H, 5.28.

3,5 - Diiodo - 4 - (2′,3′ - dimethylphenoxy)-DL-phenylalanine-N-acetyl Ethyl Ester (IVa).—3,5-Dinitro - 4 - (2′,3′ - dimethylphenoxy) - DL - phenylalanine-N-acetyl ethyl ester (4.45 Gm., 0.01 mole) dissolved in glacial acetic acid (200 ml.) was shaken for twenty minutes in the presence of Pd-on-charcoal (10%, 2.5 Gm.) and hydrogen (initial pressure 38 p. s. i.). Hydrogen uptake stopped at 96% of theoretical. Concentrated sulfuric acid (15 ml.) was added, the catalyst removed by filtration through Celite and concentrated sulfuric acid (75 ml.) added with cooling to the filtrate. The resulting solution was added over two hours to a stirred mixture of nitrosylsulfuric acid [sodium nitrite (2.1 Gm., 0.03 mole) added in small portions to sulfuric acid (200 ml.) at 50°, then diluted at 0° with glacial acetic acid (200 ml.)] at −5°. The tetrazonium mixture was stirred an additional one and one-half hours at 0°, then added to a well-stirred mixture at 25° of iodine (23 Gm., 0.09 mole) and sodium iodide (23 Gm., 0.14 mole) diluted with water (1,000 ml.) and underlayed with chloroform (500 ml.). After two hours, the chloroform phase was separated and the aqueous layer extracted with chloroform. The combined chloroform extracts were washed with 0.5 *N* sodium metabisulfite and water, dried over calcium chloride, and the chloroform removed under reduced pressure. The residue was dissolved in dry chloroform (20 ml.), passed through an alumina column (acid washed, 13 × 2.0 cm.), the eluate evaporated, and the residue crystallized from alcohol. Yield 3.5 Gm. (58%), m. p. 130–131°.

Anal.—Calcd. for $C_{21}H_{23}I_2NO_4$ (607.3): C, 41.53; H, 3.82. Found: C, 41.85; H, 3.88.

3,5 - Diiodo - 4 - (2′,3′ - dimethylphenoxy)-DL-phenylalanine (V).—3,5-Diiodo-4-(2′,3′-dimethylphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (3.0 Gm., 0.005 mole) was heated under reflux for one hour with glacial acetic acid (15 ml.) and concentrated hydrochloric acid (15 ml.). The cooled mixture was diluted with water to 100 ml., 6 *N* sodium hydroxide (30 ml.) was added, then adjusted carefully to pH 5.0 with 1 *N* sodium hydroxide. The flocculent precipitate was collected by centrifugation, washed thoroughly with water, and dried *in vacuo*. Crystallization from aqueous acetic acid yielded 2.4 Gm. (90%) of V, m. p. 197–199° (decompn.).

Anal.—Calcd. for $C_{17}H_{17}I_2NO_3$ (537.3): C, 38.01; H, 3.19; I, 47.25. Found: C, 37.88; H, 3.55; I, 47.02.

3,5 - Dinitro - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy) - DL - phenylalanine - N - acetyl Ethyl Ester (IIIb).—3,5-Dinitro-4-(*p*-toluenesulfonyloxy)-DL-phenylalanine-N-acetyl ethyl ester (52.0 Gm., 0.104 mole) was dissolved in dry chloroform (100 ml.) and heated under reflux with dry pyridine (16 ml., 0.2 mole) for forty-five minutes. 2,3-Dimethyl-4-methoxyphenol (8.0 Gm., 0.052 mole) was added and the mixture heated at reflux temperature for an additional three hours. After cooling, the chloroform solution was washed successively with 2 *N* hydrochloric acid, water, 2 *N* sodium hydroxide, and water, dried over calcium chloride, and the chloroform removed under reduced pressure. The residue was crystallized from ethanol, yielding 4.0 Gm. (50%), m. p. 142–143°.

Anal.—Calcd. for $C_{22}H_{25}N_3O_4$ (475.4). C, 55.57; H, 5.30. Found: C, 55.84; H, 5.16.

3,5 - Diiodo - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy) - DL - phenylalanine - N - acetyl Ethyl Ester (IVb).—3,5 - Dinitro - 4 - (2′,3′ - dimethyl - 4′-methoxyphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (5.0 Gm., 0.011 mole) was converted to the diiodo derivative by a procedure identical with that used in the synthesis of 3,5-diiodo-4-(2′,3′-dimethylphenoxy) - DL - phenylalanine - N - acetyl ethyl ester (IVa). Crystallization from absolute alcohol–acetone (4:1) yielded 3.5 Gm. (52%), m. p. 196–198°.

Anal.—Calcd. for $C_{22}H_{25}I_2NO_5$ (637.3): C, 41.46; H, 3.95. Found: C, 41.64; H, 4.02.

3,5 - Diiodo - 2′,3′ - dimethyl - DL - thyronine (VI).—3,5 - Diiodo - 4 - (2′,3′ - dimethyl - 4′ - methoxyphenoxy)-DL-phenylalanine-N-acetyl ethyl ester (1.5 Gm., 0.0025 mole) was heated under reflux for four hours with glacial acetic acid (15 ml.) and hydriodic acid (58%, 30 ml.). The reaction mixture was distilled to near dryness from a water bath at 50° (5 mm.). The residue was dissolved in a suspension of sodium metabisulfite in hot ethanol, and the solution adjusted to pH 5.0 with 2 *N* sodium acetate. The precipitate was collected by centrifugation, dissolved, and precipitated three times from 1 *N* sodium hydroxide by adjustment to pH 5.0 with 2 *N* hydrochloric acid and a few drops of aqueous sodium acetate, washed by suspension in water, and collected by centrifugation. Drying at 100° (2 mm.) over phosphorus pentoxide yielded 0.85 Gm. (65%) of a colorless hygroscopic powder, m. p. 223–225° (decompn.).

Anal.—Calcd. for $C_{17}H_{17}I_2NO_4$ (553.2). C, 36.91; H, 3.10; I, 45.89. Found: C, 37.27; H, 3.09; I, 46.12.

[4] Aldrich Chemical Co., Milwaukee, Wis.

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The Pharmacy of Chlorothiazide (6-Chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide): A New Orally Effective Diuretic Agent*

By WALTER F. CHARNICKI†, FREDERICK A. BACHER, STEWART A. FREEMAN, and DONALD H. DeCESARE

Chlorothiazide occurs as a white, crystalline powder which can be formulated in tablets or suspension for use as an oral diuretic. It is very slightly soluble in water and is soluble but unstable in alkaline solutions. The solid sodium salt is stable and can be dissolved in water for injection to give a suitable intravenous dosage form. Chlorothiazide's absorption maximum at 292 mμ in 0.1 *N* sodium hydroxide can be used for analysis of all dosage forms.

A RECENT STUDY of aromatic sulfonamides has led to the synthesis of benzothiadiazine derivatives (1). Pharmacological studies have revealed that this series of compounds possesses diuretic activity of rather unusual quality. In animals they not only promote the excretion of sodium but also of chloride ions (2, 3, 4). The compound selected for clinical trial from this series was 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide, known generically as chlorothiazide (Diuril). This derivative belongs to a class of heterocyclic compounds of which the parent compound is 1,2,4-benzothiadiazine.

Clinical studies in man have substantiated the earlier pharmacological findings and confirmed the unusual ability of chlorothiazide to promote the excretion of sodium and chloride, and to a smaller extent, potassium chloride. This suggested that it be designated a saluretic agent (5, 6, 7). More recently it has been observed that chlorothiazide produces significant reduction of blood pressure in hypertensive but not normotensive patients. Furthermore, it potentiates the antihypertensive action of drugs such as reserpine, veratrum alkaloids, hydralazine, and ganglionic blocking agents, and also enhances the hypotensive effects of surgical sympathectomy (8–11).

The mode of action of chlorothiazide whereby it enhances the excretion of sodium and chloride has not been fully defined. *In vitro* studies have shown the compound to have carbonic anhydrase inhibitory action which seems to be relatively specific for the renal tubular mechanism.

Chlorothiazide has a high therapeutic index and a low inherent toxicity. In human bioassays, as judged by enhancement of sodium excretion, chlorothiazide in a dose of 0.25 Gm. orally has an effect comparable to that of the classic carbonic anhydrase inhibitors. An oral dose of 0.5 to 1.0 Gm. of chlorothiazide is comparable to the oral administration of mercurial diuretics, and an oral dose of 1.0 to 2.0 Gm. to parenteral mercurials. Chlorothiazide thus has wide application.

The onset of action is within two hours following the oral administration of chlorothiazide with maximal effect at about four hours. The major saluretic effect lasts approximately six to twelve hours.

It is of interest to note that following intravenous administration of chlorothiazide the onset of action is within fifteen minutes, the maximal effect being obtained within thirty minutes. The duration of effect is relatively short; the major response lasts about two hours. The total effect upon electrolyte and water excretion is thus brief and quantitatively less than that of a comparable oral dose.

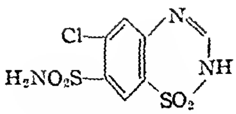
The usual adult dosage range is one or two 0.5-Gm. tablets of chlorothiazide once or twice a day. When the total daily dose is 1.0 Gm. or more it is usually administered in two divided doses at an interval of six to twelve hours.

The clinical reports received to date attesting to the outstanding value of chlorothiazide as a safe and effective agent in the treatment of edematous states as well as in the treatment of hypertensive vascular diseases have prompted us to describe the physical properties and pharmaceutical dosage forms of this new diuretic agent.

PHYSICAL PROPERTIES

Chlorothiazide is a white crystalline compound melting about 355° with decomposition. Its empirical formula is $C_7H_6ClN_3O_4S_2$ and the molecular weight is 295.7. It may be represented structurally as follows:

* Received June 5, 1959, from the Merck Sharp & Dohme Research Laboratories, Division of Merck & Co., Inc., West Point, Pa.
† Present address: Smith-Dorsey Pharmaceuticals, Lincoln 1, Nebr.



Chlorothiazide

Chlorothiazide is very slightly soluble in water and glycerin. It is practically insoluble in ether, chloroform, and benzene. One gram dissolves in about 300 ml. of acetone, 500 ml. of 95% ethanol, 350 ml. of methanol, 150 ml. of pyridine, or 9 ml. of dimethylformamide. The solubility of chlorothiazide in water or dilute acids is approximately 0.2 mg. per ml. At pH 7 the solubility is approximately 0.7 mg. per ml. and at pH 8.5 it is 16 mg. per ml. More than 1 Gm. will dissolve in 10 ml. of 1 N sodium hydroxide.

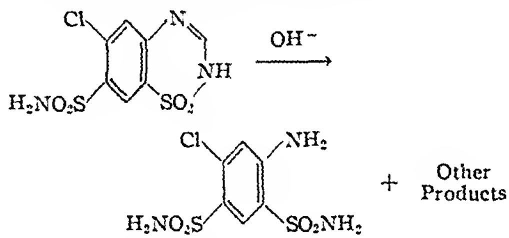
Chlorothiazide is a weak dibasic acid with $pK_1 = 6.7$ and $pK_2 = 9.5$. In acid solution it shows an ultraviolet absorption maximum at 278 mμ, with a (1%, 1 cm.) approximately 420. In 0.1 N sodium hydroxide the absorption maximum occurs at 292 mμ, with a (1%, 1 cm.) approximately 430.

The heterocyclic ring of chlorothiazide is slowly hydrolyzed in alkaline solutions yielding a diazotizable amine. At room temperatures, solutions in 0.1 N sodium hydroxide hydrolyze at the rate of approximately 1% per hour while at 100° the initial rate is about 2% per minute. While the stability of chlorothiazide in neutral or acidic solutions is substantially greater, the low solubility of the drug at lower pH's precludes the attainment of physiologically effective concentrations.

TYPICAL FORMULATIONS

Compressed Tablets.—Stability studies have indicated that compressed tablets of chlorothiazide are satisfactory when manufactured by either the wet granulating or by "slugging" techniques. Tablets containing 250 mg. and 500 mg. of chlorothiazide and corn starch, and granulated with starch paste have been made with a hardness of 7 to 8 Kg. and a disintegration time of five minutes at 37°. Tablets containing 250 mg. of chlorothiazide and additives such as polyethylene glycol 4000 and methylcellulose to increase hardness have been made by slugging. These usually have a hardness of 4 to 5 Kg. and a disintegration time of five minutes at 37°. Hardness was determined with a Monsanto hardness tester and disintegration was taken with a Stoll-Gershberg type of apparatus.

Injection.—Chlorothiazide is uniformly and well absorbed when given orally. However, when oral administration is not possible or desirable, chlorothiazide may be administered intravenously. The dosage form of chlorothiazide for intravenous use requires the chlorothiazide to be either in solution or in a form completely and quickly soluble. Chlorothiazide is somewhat soluble in polyethylene glycol 300 and polyethylene glycol 400. It is readily soluble in dilute sodium hydroxide solutions. The latter solutions, unfortunately, decompose upon standing at room temperature or elevated temperatures with hydrolysis of the heterocyclic ring. The reaction may be summarized as follows:



Because of this instability sterile freeze-dried solids were investigated for parenteral use in clinical studies. Aqueous solutions of the monosodium salt of chlorothiazide freeze-dry to yield hard shrunken plugs with a poor appearance and which dissolve very slowly upon adding water. This difficulty was overcome by the addition of suitable excipients for lyophilization, particularly dry sugar alcohols. Freeze-dried solids containing 0.5 Gm. of chlorothiazide as the sodium salt can be restored with 10 ml. or more of water for injection to give a clear solution having a pH of 9.5. When a vial containing 0.5 Gm. of chlorothiazide (as the sodium salt) is restored with 20 ml. of water, the resulting solution is practically isotonic and has a freezing point of −0.505°.

Vials of freeze-dried chlorothiazide may also be restored with normal saline and 5% dextrose.

TABLE I.—COMPATIBILITY OF CHLOROTHIAZIDE WITH HYPOTENSIVE AGENTS

Drug[a]	pH	Amount Added,[b] ml.	pH of Mixture	Appearance[c] Initially	After 24 Hr.
Protoveratrines A and B	5.9	1.2	9.1	S	S
Pentolinium tartrate	5.3	1.0	9.1	S	S
Hydralazine hydrochloride	3.7	2.0	9.0	H	P
Cryptenamine acetate	4.7	1.0	9.2	S	S
Alkavervir	3.2	1.0	9.0	S	S
Reserpine	3.0	1.0	9.0	H	P
Mecamylamine hydrochloride	7.5	1.0	9.2	S	S
Cryptenamine acetate	4.7	1.0			
plus hydralazine hydrochloride	3.7	2.0	9.0	H	P
Reserpine	3.0	1.0			
plus hydralazine hydrochloride	3.7	2.0	8.8	H	P
Reserpine	3.3	0.5			
plus mecamylamine HCl	7.5	1.0	9.3	H	P

[a] Hypotensive agent in commercially available solution.
[b] The indicated volume of solution was added to 125 ml. of 0.4% chlorothiazide solution in 5% dextrose. The pH of this solution was 9.15.
[c] S—Solution clear with no discoloration or precipitate. H—Solution became cloudy and developed color. P—Precipitate developed.

These diluents do not alter the pH and therefore result in clear solutions. On the other hand, diluents which lower the pH of the restored solution to pH 8.8 or less tend to yield cloudy solutions due to precipitation of chlorothiazide free acid.

A 10% solution of chlorothiazide free acid may be prepared in a 75% aqueous solution of polyethylene glycol 300 or 400. Although this solution is effective as a diuretic when administered intravenously to the dog, clinical studies were not conducted.

Compatibility of Sodium Chlorothiazide Solutions With Hypotensive Drugs.—The potentiating action of chlorothiazide upon such hypotensive drugs as reserpine, veratrum alkaloids, hydralazine, and ganglionic blocking agents led us to study the compatibility of chlorothiazide sodium solution with some of these agents. The results of this study are shown in Table I.

Oral Fluid Preparation.—Chlorothiazide free acid is only very slightly soluble and is therefore quite stable in aqueous suspension at pH 6.5 or lower. It is also compatible with such materials as tragacanth, carboxymethylcellulose, methylcellulose, gelatin, and acacia. Suspensions prepared with tragacanth in a syrup base were found to be quite satisfactory both physically and chemically. The particle size of the chlorothiazide used in the suspensions averaged 5 μ and the preparations resuspended very readily with no evidence of caking even after storage at higher temperatures. They were readily flavored to mask the slight bitterness of the chlorothiazide by the addition of citrus flavors.

Stability.—The stability of chlorothiazide in the formulations described was evaluated over prolonged intervals at widely varying temperatures. The excellent stability of chlorothiazide in these products has been summarized in Tables II, III, and IV.

ANALYTICAL PROCEDURES

Chlorothiazide can be assayed by ultraviolet absorption at 292 mμ after diluting the sample with 0.1 N sodium hydroxide, filtering if necessary, and then diluting to approximately 1 mg. per 100 ml. for

TABLE II.—STABILITY OF CHLOROTHIAZIDE IN TABLET FORMULATIONS

Formulation	Storage Temp. °C.	Storage Period	Assay % Label Claim
C.T. 500 mg.[a]		Initial	101
	80	5 days	99
	70	10 days	101
	60	20 days	99
	37	2 mo.	101
		4 mo.	98
		6 mo.	98
		13 mo.	98
	RT	4 mo.	99
		6 mo.	99
		13 mo.	101
C.T. 250 mg.[b]		Initial	99
	50	1 mo.	94
	37	7$^1/_2$ mo.	99
	RT	4$^1/_2$ mo.	99
		7$^1/_2$ mo.	100

[a] Prepared by wet granulation.
[b] Prepared by slugging.

TABLE III.—STABILITY OF CHLOROTHIAZIDE IN FREEZE DRIED PREPARATION

Storage Temp. °C.	Storage Period	Assay % Label Claim
	Initial	98
60	2 wks.	94
50	2 wks.	98
	5 mo.	95
	14 mo.	99
37	1 mo.	96
	5 mo.	98
	14 mo.	99
RT	2 mo.	101
	5 mo.	100
	14 mo.	100

TABLE IV.—STABILITY OF LYOPHILIZED CHLOROTHIAZIDE 500 MG. AFTER RESTORATION

Diluent	Volume ml.	Assay % Initial Concentration — Time After Restoration Hr. 0	3	22	48	72
Water for injection	10	100	99.5	98	97.5	98
Dextrose 5%	10	100	99.5	98	97	96
Dextrose 5%	500	100	99.5	98	94	94

measurement. If the measurement is completed within one hour, alkaline decomposition is insignificant. Many neutral or basic interferences can be removed by washing an alkaline solution with chloroform before final dilution. Acidic or phenolic substances can usually be removed by washing an intermediate acidic dilution (less than 30 mg./100 ml.) with chloroform before final dilution with 0.1 N sodium hydroxide.

While the major hydrolysis product of chlorothiazide (see reaction for decomposition) is not removed by the above treatments, the absorption of this decomposition product at 292 mμ is only 25% that of chlorothiazide. For stability studies in which apparent decomposition (as determined by ultraviolet absorption) is less than 10%, no significant error is introduced if the absorption due to the amine is ignored. The hydrolysis product itself can be determined by diazotization and coupling by a modified Bratton Marshall method (12).

Compressed Tablets.—Weigh accurately powdered tablets equivalent to 250 mg. of chlorothiazide into a 250 ml. volumetric flask. Add about 200 ml. 0.1 N sodium hydroxide solution and shake for fifteen minutes. Dilute to volume with sodium hydroxide solution, *mix thoroughly, and filter about* 50 ml. through a No. 2 Whatman paper discarding the first 25 ml. of filtrate. Dilute 1 to 100 ml. with 0.1 N sodium hydroxide and determine absorbance at 292 mμ. Calculate using a (1%, 1 cm.) = 430.

Freeze-Dried Sodium Salt.—Dissolve the contents of one vial (approx. 500 mg.), transfer quantitatively to a 250 ml. volumetric flask with 0.1 N sodium hydroxide, and dilute to volume. Dilute this further with 0.1 N sodium hydroxide, 25 ml. to 500 ml. and 10 ml. of the latter solution to 100 ml. Determine absorbance at 292 mμ and calculate using a (1%, 1 cm.) = 430.

Oral Suspension.—Dilute 5 ml. of suspension to 250 ml. with 0.1 N sodium hydroxide (solution A). Dilute 10 ml. of solution A to 100 ml. with 0.1 N

hydrochloric acid (solution B) Wash about 50 ml of solution B with 2 × 25 ml chloroform Dilute 10 ml of washed solution B to 100 ml with 0 1 *N* sodium hydroxide Read absorbance at 292 mμ and calculate using *a* (1%, 1 cm) = 430

SUMMARY

1 Methods were developed for the preparation of compressed tablets, suspension, and freeze-dried solid of chlorothiazide

2 Analytical procedures were developed for the assay of chlorothiazide in these dosage forms

3 The chlorothiazide in these dosage forms has been found to be quite stable in both accelerated and long term stability studies

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Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don.) V*

Preparation and Characterization of Alkaloids

By GORDON H. SVOBODA, NORBERT NEUSS, and MARVIN GORMAN

As part of continuing study of alkaloid-bearing members of the family *Apocynaceae,* we have undertaken a detailed phytochemical investigation of the pantropical plant *Vinca rosea* Linn. (1). Twelve crystalline compounds have been obtained and characterized. Details of the procedure leading to the preparation of pure substances are described. Two of these alkaloids, leurosine and vincaleukoblastine, possess activity against P-1534 leukemia in mice.

THE APOCYNACEOUS plant *Vinca rosea* Linn has enjoyed a wide reputation in folk medicine as an oral hypoglycemic agent[1] (2, 3) As part of our general program of investigation of plants in this family (4), a phytochemical study of this plant was undertaken Various alkaloidal and nonalkaloidal fractions were tested for their ability to lower blood sugar In our hands, however, these results were all negative (5)[2] During the testing, the observation was made that certain fractions prepared from the crude drug produced a delayed toxicity in experimental animals Subsequently these extracts were submitted for testing against experimental leukemia (10) Limited prolongation of life was observed in DBA-2 mice against P-1534 leukemia with certain preparations of plant material

This activity prompted a systematic fractionation of extracts in a search for the active principles free of extraneous, undesirable toxic substances Initial testing soon indicated that the biologically active entities were confined to the complex alkaloidal portion of the drug

In this communication a procedure is described for the preparation of leurosine and vincaleukoblastine,[3] two alkaloids possessing the anti-

* Received August 21, 1959 from the Organic Chemical Development and Lilly Research Laboratories, Eli Lilly and Co, Indianapolis, Ind

We wish to acknowledge and thank the following individuals for their aid in the course of this investigation Botanical classification Dr Julian Steyermark Research Associate Missouri Botanical Gardens Plant processing Mr A J Barnes, Jr Laboratory assistance Mrs Nancy J Cone, Miss Ruthanne Miller, Messrs R J Armstrong, A T Oliver, and George Johnson Physical data Miss Ann Van Camp, Dr H E Boaz, Mr L G Howard, and associates Microanalyses Messrs G M Maciak, W L Brown, H L Hunter and R Hughes Biological evaluations Drs M Root and I S Johnson and associates Paper chromatography Mr H Bird Plant procurement Ellis Meer Corp, New York, N Y Alkaloid samples Dr C T Beer, University of Western Ontario, London Canada, authentic sample of vincaleukoblastine sulfate Professor Janot and Dr Jean Le Men, University of Paris, Faculty of Pharmacy, Paris France, sample of vindolinine dihydrochloride

Presented to the Scientific Section, A PH A, Cincinnati meeting August 1959

[1] In an earlier description of medicinal properties of this plant, Peckolt has mentioned that the infusion of the leaves of *Vinca rosea* Linn is used in Brazil against hemorrhage, scurvy, as a mouthwash for toothache and for healing and cleaning of chronic wounds (6) Most recently Chopra, *et al* have reported that the total alkaloids possess a limited antibiotic activity (against *V cholera* and *M pyogenes* var *aureus*) as well as a significant and sustained hypotensive action (6a)

[2] A literature search revealed that earlier workers have investigated the properties of this plant on carbohydrate metabolism with essentially negative results (7, 8, 9)

[3] After the preparation of leurosine by one of us (G H S) it was learned that Drs Noble, Beer, and Cutts of the Department of Medical Research University of Western Ontario, London Canada have also observed hematological responses in certain preparations of *Vinca rosea* Linn leaves and subsequently have obtained an active alkaloid vincaleukoblastine Since that time certain phases of the work on vincaleukoblastine have been carried out in cooperation with the Canadian group.

Fig 1 —*Vinca rosea* Linn

leukemic activity mentioned above Pertinent data regarding other crystalline alkaloids (Tables I and II) obtained from this plant are also presented

BOTANICAL AND CHEMICAL CHARACTERISTICS

Botanical Description of Vinca rosea Linn—*Vinca rosea* Linn is an erect, everblooming pubescent herb or subshrub, one to two feet high (Fig 1) (11) It is generally cosmopolitan in the tropics but is also cultivated widely in gardens throughout the world (12) Two color varieties (pink and white) are found in the natural state and an additional three (blush pink with red eye, crimson, and white with red eye) are listed as commercially available seed hybrids (13) Generally accepted synonyms for the genus *Vinca* are *Pervinca*, *Lochnera*, and *Catharanthus* [4] The genus is placed in the group *Lochnerineae* of the tribe *Alstoniae*, of the sub family *Plumeroideae* [4]

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[4] Pichon an authority in the taxonomy of the *Apocynaceae* has shown however that the genera *Vinca* and *Lochnera* differ in thirty four morphological characteristics and therefore should not be used as synonyms He also found that the name *Lochnera Reichb* was predated some seven months by the name *Catharanthus* G Don Therefore the correct botanical name for this plant is *Catharanthus roseus* G Don (14) but to avoid confusion we shall continue to use the more common *Vinca rosea* Linn A complete botanical description and bibliography on the genus *Vinca* Linn is given in the doctoral thesis of Marcelle Gabbai Les Alcaloides des Parvenches *Vinca et Catharanthus* (*Apocynaceae*) Universite de Paris Faculte de Pharmacie Serial No U 291 1958 Paris France We thank Dr Jean Le Men of this university for making the thesis available to us

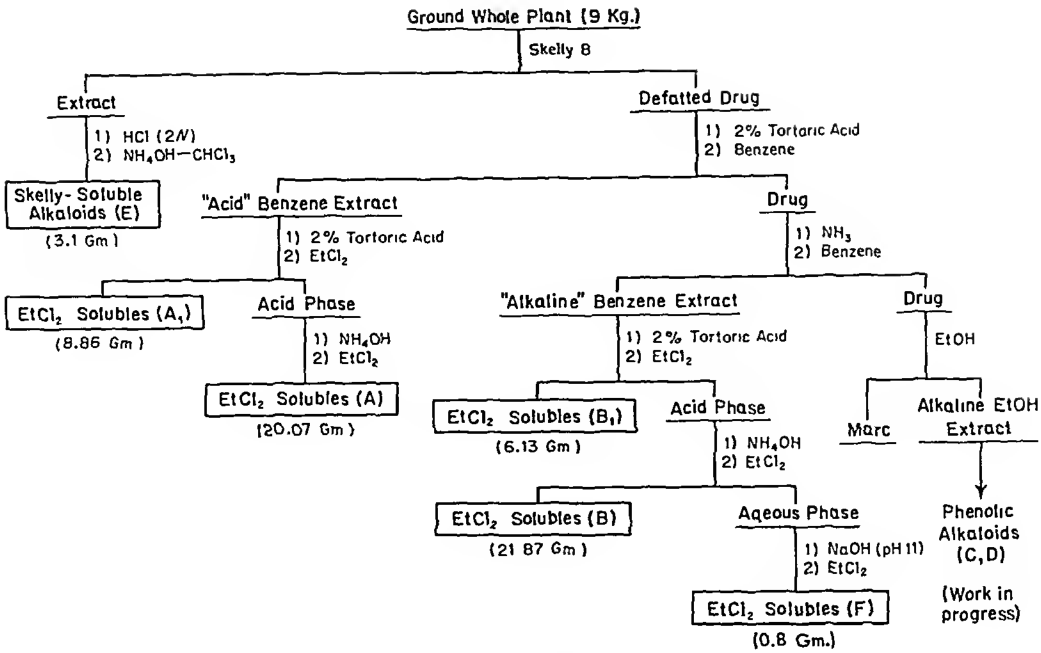


Fig. 2.—Extraction scheme

TABLE I.—ALKALOIDS FROM *Vinca rosea* LINN.

No	Name	Formula
1	Ajmalicine	$C_{21}H_{24}O_3N_2$
2	Tetrahydroalstonine	$C_{21}H_{24}O_3N_2$
3	Serpentine	$C_{21}H_{22}O_3N_2$
4	Lochnerine	$C_{20}H_{24}O_2N_2$
5	Reserpine[a]	$C_{33}H_{40}O_9N_2$
6	Akuammine[a]	$C_{22}H_{26}O_4N_2$

[a] Reserpine and akuammine (vincamajoridine) have not been obtained during this work

Alkaloidal Constituents of Vinca rosea Linn.—The earliest chemical investigation of the plant was carried out by M. Greshoff (15), who indicated the presence of alkaloidal constituents but was unable to obtain homogenous, crystalline compounds An advance in the phytochemistry of this plant was made by Cowley and Bennett (11) in 1928 when they were able to prepare two crystalline sulfates and a tartrate from the total alkaloidal fraction; however, no chemical or physical properties of these salts were described. In 1953, R. Paris and Moyse-Migmon reported an unidentified crystalline alkaloid from this plant (16). More recently several groups have obtained the following alkaloids (Table 1): ajmalicine (17), akuammine[5] (17), tetrahydroalstonine (18), serpentine (17, 18), lochnerine (19), and reserpine[5] (20)

While our investigation was in progress, Kamat and co-workers (21) have reported on two amorphous and two crystalline alkaloids Only one of these was described in detail and named vindoline At about the same time Noble, Beer, and Cutts have described the alkaloid vincaleukoblastine (22) and one of us (G H S) obtained three additional alkaloids, leurosine, perivine, and virosine (23) Most recently we have given details of the characterization of additional new *Vinca* alkaloids, *viz*, catharanthine, vindolinine,[6] and lochnericine[7] and confirmed the presence of the compounds reported earlier (25) (Table II)

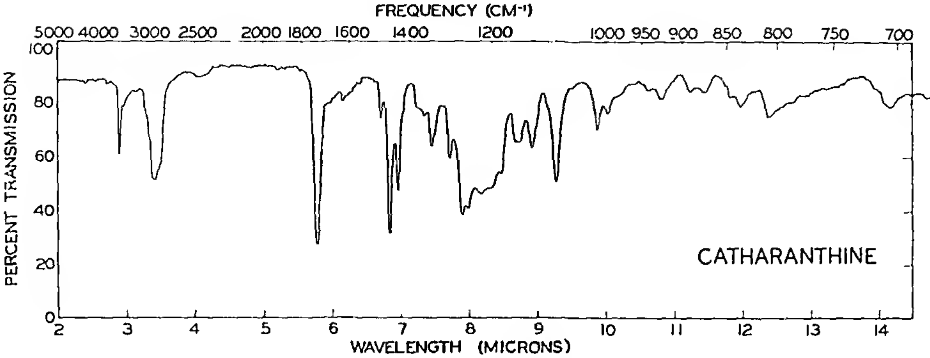
The alkaloids reported prior to 1958 (Table I) are well documented and do not merit further discussion The remaining alkaloids are listed in Table II The empirical formulas, m p 's, pKa's, optical rotations, and ultraviolet absorption maxima are also given As an additional aid to the characterization of these alkaloids, their respective infrared spectra are reproduced separately (Fig 3) The characterization of alkaloids 1–6 has been described in detail by us previously (25, 26). Perivine and virosine have been reported by one of us (G. H S) but no empirical formulas have been assigned (23) Perivine is now shown to be a $C_{20}H_{24}O_3N_2$ compound This formulation was corroborated by the analysis of the hydrochloride as well as the determination of the molecular weight of this salt from X-ray data measurements of the unit cell The spectral data of perivine indicate that this compound is another representative of 2-acyl indole alkaloids (27) Virosine has been tentatively formulated as a $C_{22}H_{26}O_4N_2$ compound Its spectral characteristics are as yet uninterpreted and small amounts of the alkaloid did not allow any degradative studies

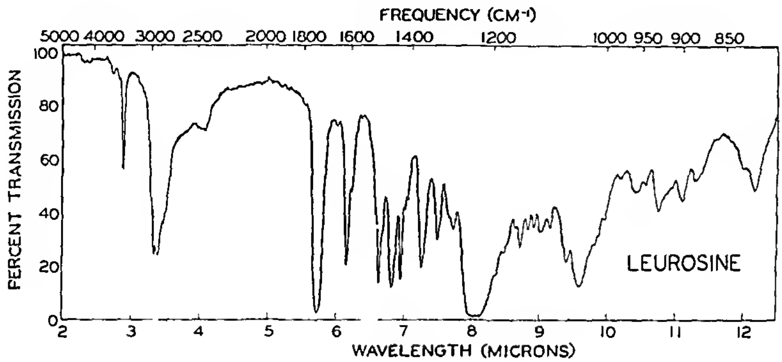
Phytochemical Analysis of the Alkaloids of Vinca rosea Linn.—Figure 2 represents a brief outline of the scheme used in the preparation of the alkaloids The process consists essentially of separating the plant alkaloids into those whose tartrates are soluble in organic solvents and those which are insoluble under these conditions In each fraction (A_1, A, B_1,

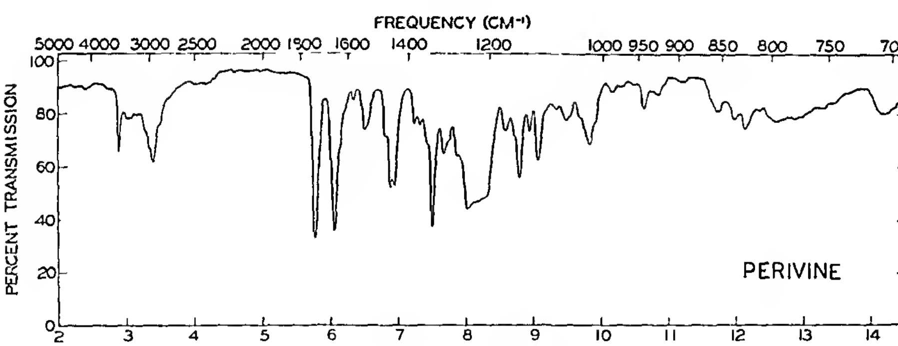
[5] These alkaloids were not encountered during our investigation

[6] The occurrence of this alkaloid has been privately communicated to us by Professor Janot, University of Paris and the name was chosen by mutual agreement with his group

[7] An alkaloid, independently named lochnericine, has been reported by C P N Nair and P P Pillay (24) This material appears to be identical with our lochnericine.







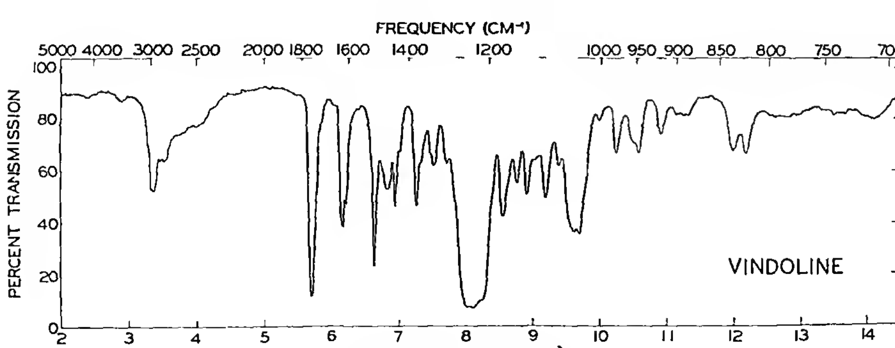


Fig 3.—Infrared spectra of alkaloids The infrared spectrum of vindolinine dihydrochloride was obtained in a mineral oil mull. All other spectra were recorded in chloroform in a 0.1 mm. path, using the Perkin-Elmer Model 21 spectrophotometer.

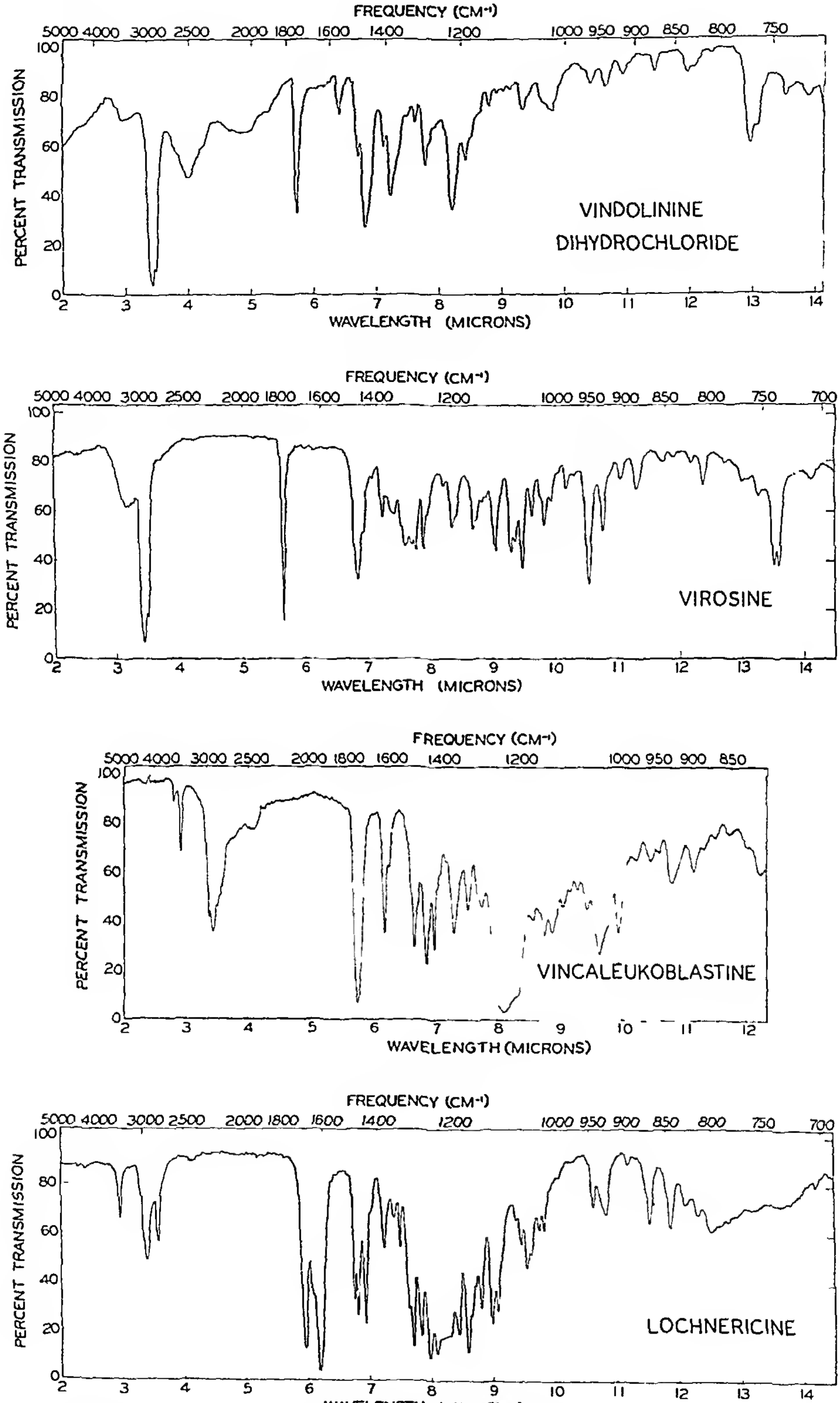


Fig. 3.—Infrared spectra of alkaloids. The infrared spectrum of vindolinine dihydrochloride was obtained in a mineral oil mull. All other spectra were recorded in chloroform in a 0.1 *mm.* path, using the Perkin-Elmer Model 21 spectrophotometer

B, etc.) the final purification and eventual separation of biological activity is achieved by careful elution chromatography on deactivated alumina. Details of this procedure are described in the experimental portion of this paper.

Preliminary observations pertaining to the interrelationship of the biologically active alkaloids leurosine and vincaleukoblastine with the indole alkaloid catharanthine and the dihydroindole alkaloid vindoline have been reported elsewhere (1). At the present time we are continuing the investigations toward a complete structure elucidation of these substances.

EXPERIMENTAL

All known alkaloids isolated above were identified by comparison of mixed melting point, infrared spectra, and X-ray diffraction patterns with those of authentic specimens (28). The melting points were determined on a Kofler microstage.

The alkaloidal extracts were obtained using the following procedure. Ground whole plant (9 Kg.) was defatted by stirring with two 45-L. portions of Skelly B. This extract gave a (+) Mayer's test. Concentration to 3 L. and extraction with 2 *N* HCl (4 × 200 cc.) gave, after treatment of the acid with NH_4OH and ether extraction, 3.1 Gm. of alkaloids (E).

The defatted drug was intimately mixed with 6 L. of 2% tartaric acid and extracted by stirring with three 48-L. portions of benzene. The combined extracts were concentrated *in vacuo* to approximately 9 L. The alkaloids were extracted from this benzene concentrate with 2% tartaric acid (two 12-L. portions). The acid extract was then extracted at pH 3.2 with two 6-L. portions of ethylene dichloride. Drying and evaporation yielded 8.86 Gm. (A_1).

The acid solution was made alkaline to litmus with NH_4OH and was extracted with 6 L. of ethylene dichloride. The solution was dried and evaporated to give 20.07 Gm. (A).

After the "acid" benzene extract had been collected, NH_3 was bubbled through the drug (in benzene) until it was alkaline. The alkalinized drug was extracted by stirring with three 48-L. portions of benzene. The "alkaline" benzene extract was processed in the same manner as was that from the "acid" benzene. The yields of (B_1) and (B) were 6.13 Gm. and 21.87 Gm., respectively. After removal of the B fraction, the pH of the ammoniacal solution was adjusted to pH 11 with sodium hydroxide pellets. Extraction of this solution with chloroform followed by drying and evaporation gave 0.8 Gm. of alkaloid (F).

The alcoholic extract of the ammoniacal marc has yielded a number of phenolic bases which are presently being investigated (C, D).

Benzene solutions of 10 Gm. of each of these materials, obtained as amorphous powders, were subsequently chromatographed on 400 Gm. of alumina (Alcoa alumina, Grade F-20) deactivated by treatment with 12.5 cc. of 10% acetic acid in the usual manner. The eluting solvents were benzene, benzene-chloroform mixtures, chloroform, and finally chloroform-methanol mixtures. Fractions were collected, evaporated *in vacuo*, and combined, where indicated, by infrared spectroscopy. The purified alkaloids were crystallized from a suitable solvent or converted to an appropriate salt. The results are shown in Tables III, IV, V, VI, VII, and VIII.

Characterization of Perivine and Virosine.—*Perivine.*—From the chromatography of 20 Gm. of fraction B (Table VI) there were obtained 250 mg. of crude perivine by the elution with benzene-chloroform mixtures (1:1) (fraction 20-21). Crystallization from acetone afforded 140 mg. of crystalline perivine. Two recrystallizations from methanol gave long prisms, m. p. 218-221° (decompn.), $[\alpha]_D^{26} = -121.4°$ (C = 1, $CHCl_3$).

Anal.—Calcd. for $C_{20}H_{22}O_3N_2$: C, 70.98; H, 6.55; N, 8.28; mol. wt., 338.39. Found: C, 70.94; H, 6.77; N, 7.93; mol. wt., 355 ± 10.

pK'a 7.5, electrometric titration, 66% DMF. The hydrochloride salt was prepared in a conventional manner and recrystallized from methanol-ether.

Anal.—Calcd. for $C_{20}H_{22}O_3N_2 \cdot HCl$: C, 63.74; H, 6.69; N, 7.43; Cl, 9.41; mol. wt. 374.86. Found: C, 63.89; H, 6.71; N, 7.51; Cl, 9.33; mol. wt. 373.5 ± 1% (X-ray). λ_{max}^{EtOH} 226 mμ (*E* = 19,100) and 314 mμ (*E* = 19,200).

Virosine.—A careful chromatography of 50 Gm. of the fraction A (Table IV) yielded 55 mg. of pure virosine which, after two recrystallizations from chloroform-methanol, afforded long prisms, m. p. 258-261° (decompn.), $[\alpha]_D^{26} = -160.5°$ (C = 1, $CHCl_3$).

TABLE II.—ALKALOIDS FROM *Vinca rosea* LINN.

No.	Name	Formula	M. p., °C.	$[\alpha]_D^{26}$ $CHCl_3$	pK'a in 66% DMF	U. V. λ_{max}^{EtOH} in mμ	Lit. Ref.
1	Vindoline	$C_{25}H_{32}O_6N_2$	154-155	+42	5.5	212, 250, 304	25
2	Catharanthine	$C_{21}H_{24}O_2N_2$	126-128	+29.8	6.8	226, 284, 292	25
3	Vindolinine 2HCl	$C_{21}H_{24-26}O_2N_2 \cdot 2HCl$	210-212 (decompn.)	−8 (H_2O)	3.3, 7.1	245, 300	25
4	Lochnericine	$C_{21}H_{24}O_3N_2$	190-193 (decompn.)	−432	4.2	226, 297, 327	24, 25
5	Leurosine	$C_{46}H_{58}O_9N_4$	202-205 (decompn.)	+72	5.5, 7.5 (H_2O)	214, 259	23, 26
6	Vincaleukoblastine	$C_{46}H_{58}O_9N_4$	211-216 (decompn.)	+42[a]	5.4, 7.4 (H_2O)	214, 259	22, 26
7	Perivine	$C_{20}H_{22}O_3N_2$	180-181	−121.4	7.5	226, 314	23 and this paper
8	Virosine	$C_{22}H_{26}O_4N_2$	258-264 (decompn.)	−160.5	5.85	226, 270	23 and this paper

[a] Determined on vincaleukoblastine etherate [$C_{46}H_{58}O_9N_4 \cdot (C_2H_5)_2O$].

TABLE III.—CHROMATOGRAPHY OF (A_1)

Fraction, 500 cc ea	Eluting Solvent	Compound	Wt, Gm	Crystallizing Solvent
1–2	Benzene	Amorphous residues		
3–4	Benzene	Tetrahydroalstonine	0 112	Methanol
5–8	Benzene	Amorphous residues		
9–14	Benzene-chloroform (3·1)	Vindoline	2 5	Ether
14–on	Chloroform	Amorphous residues		

TABLE IV.—CHROMATOGRAPHY OF (A)

Fraction, 500 cc ea	Eluting Solvent	Compound	Wt, Gm	Crystallizing Solvent
1	Benzene	Catharanthine	0 250	Methanol
2	Benzene	Vindolinine (as dihydrochloride)	0 210	Methanol-ether
3–19	Benzene	Ajmalicine	0 798	Methanol
20–21	Benzene	Vindoline	0 820	Ether
34–42	Benzene-chloroform (1·1)	Leurosine	0 234	Methanol
43–45	Benzene-chloroform (1:1)	Vincaleukoblastine (as sulfate)	0 126	Ethanol
46	Chloroform	Virosine	0 010	Acetone
47–52	Chloroform-methanol	Amorphous residues		

TABLE V.—CHROMATOGRAPHY OF (B_1)

Fraction, 500 cc ea	Eluting Solvent	Compound	Wt, Gm	Crystallizing Solvent
1–2	Benzene	Amorphous residues		
3	Benzene-chloroform (3·1)	Ajmalicine[a]	0 059	Acetone
4–11	Benzene-chloroform (1·1)	Amorphous residues		
12–13	Benzene-chloroform (1·1)	β-Sitosterol	0 008	Methanol
14–on		Amorphous residues		

[a] Isolated as a monohydrate

TABLE VI —CHROMATOGRAPHY OF (B)

Fraction, 650 cc ea	Eluting Solvent	Compound	Wt, Gm	Crystallizing Solvent
1–11	Benzene	Oily residues		
12–13	Benzene-chloroform (3.1)	Ajmalicine	0 055	Methanol
14–19	Benzene-chloroform (3·1)	Amorphous residues		
20–21	Benzene-chloroform (1:1)	Perivine	0 084	Acetone
22–24	Benzene-chloroform (1.1)	Amorphous residues		
25–27	Benzene-chloroform (1·1)	Lochnerine	0 581	Acetone
28–38	Chloroform	Amorphous residues		
39	Methanol	Amorphous residues		

TABLE VII —CHROMATOGRAPHY OF (E), 3 GM

Fraction, 150 cc ea	Eluting Solvent	Compound	Wt, Gm	Crystallizing Solvent
1–3	Benzene	Yellow oil		
4–6	Benzene	Lochnericine	0 23	Methanol
7–18	Benzene	Oils		
19–28	Benzene	Tetrahydroalstonine	0 08	Methanol
29–53	Benzene-chloroform (3 1)	Oil		
54–58	Benzene-chloroform (3 1)	Vindoline	0 43	Ether

TABLE VIII —CHROMATOGRAPHY OF (F), 0 8 GM

Fraction, 100 cc ea	Eluting Solvent	Compound	Wt, Gm	Crystallizing Solvent
1–10	Benzene-chloroform (1 1)	Amorphous residues		
11	Chloroform	Serpentine (as nitrate)	0 02	Water-ethanol
12	Chloroform-methanol	Amorphous residues		

Anal—Calcd for $C_{22}H_{26}O_4N_2$: C, 69 09; H, 6 85, N, 7 33; O, 16 73; mol wt, 382 44 Found: C, 68 85, 69 22, H, 6 64, 6 97, N, 7 23, O, 16 99, mol wt 349 ± 20

pK'a 5 85, electrometric titration, 66% DMF λ_{max}^{EtOH} 226 mμ (E = 29,400) and 270 mμ (E = 8,500)

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The Biological Action of Cellular Depressants and Stimulants II*

The Kinetics of the Action of Urethane on the Growth of *Tetrahymena pyriformis*

By JOHN J. EILER, JOSEPH Z. KREZANOSKI†, and KWAN-HUA LEE

A kinetic analysis of the action of urethane on growth shows that the rate of cell division of *Tetrahymena pyriformis*, both in the presence and in the absence of drug, is best described by a second-order rate law. The concentration of some rate-limiting factor critical to growth is lowered both by urethane and oxygen want.

In the first paper in this series (1), it was shown that increasing concentrations of urethane cause a progressive increase in the mean generation time of normal cells of *Tetrahymena pyriformis* Cells which have been subcultured frequently in the presence of the drug prior to the test to produce trained cells, respond to the drug in a similar fashion, but with a decrease in sensitivity. In the tests on both types of cells (normal and trained), the quantitative estimation of the effect of the several concentrations of drug was based on the rate of cell division only during the period of logarithmic growth Since logarithmic growth accounted for no more than 10 per cent of the total increase in population, it seemed proper to extend the examination of the data to seek both a more encompassing measure of drug action and, if possible, additional information concerning the mechanism of action. Such an examination is presented here

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DATA, CONCEPTS, AND DISCUSSION

The growth data which form the basis of this paper have been taken from our previous report (1) The cells were grown in 2,000-ml Provitsky flasks containing either 750 ml of a proteose-peptone-yeast extract medium or 750 ml of the medium to which had been added known amounts of urethane The flasks were inoculated with *Tetrahymena pyriformis* either from cultures of normal cells or from cultures of trained cells so as to yield, in either case, an initial population of 1,000 cells/ml The cultures were stored in the dark at 27° without agitation except for the periodic withdrawal of samples for the purpose of estimating the cell population

The increases in cell count in the absence and in the presence of the several concentrations of urethane are presented in Fig 1, where the ratio of the time-dependent population (Pt) to the initial population (Po) is plotted on a logarithmic scale *versus* time The slope of the linear portion of each of the curves constitutes a quantitative measure of the effect of a given concentration of

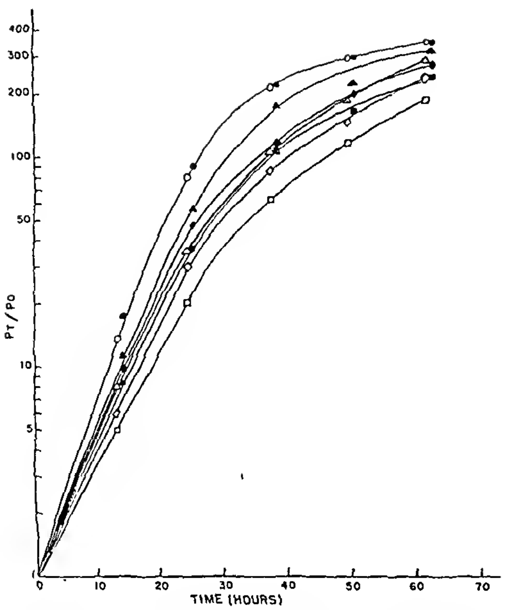

Fig. 1.—The effect of several concentrations of urethane on trained and normal cells. The time-dependent cell count (P_t) divided by the initial cell count (P_0) is plotted on a logarithmic scale. Normal cells, ○ = control; △ = 0.07 *M;* ◇ = 0.08 *M;* and □ = 0.09 *M.* Trained cells, ● = control; ▲ = 0.07 *M;* ◆ = 0.08 *M;* and ■ = 0.09 *M.*

drug. The slopes are reported as first-order reproduction constants (k_1) in Table I.

It is to be noted in each of the curves of Fig. 1 that adherence to first-order kinetics is limited to about the first five generations. A continual decrease in the reproduction constant (slope) is observed to take place after the attainment of a population of about 20,000–25,000 cells/ml. About the same limit to exponential growth seems to hold in all cases. Since the maximum populations (Px) attained ranged between 275,000–363,000 cells/ml. (see Table I), it is clear that nonexponential growth represents over 90% of the increase in cell population.

The downward concavity of the growth curve, when plotted in accordance with first-order kinetics, suggests the possibility that growth may conform to a rate law of some higher order. Growth curves following a second-order rate law (2, 3, 4) or even higher (3, 5) have been reported frequently. Indeed, Browning, *et al.* (6), have observed conditions under which *Tetrahymena* cells may grow according to second-, third-, or higher order kinetics.

After the trial of several possible rate laws, success was achieved with the use of the rate law applicable to second-order autocatalytic reactions (4, 7) which, using the symbols employed by Browning, *et al.* (5), describes the time-dependent increase in cell number as follows:

$$P_t = \frac{P_x}{1 + \frac{P_x - P_0}{P_0} \cdot e^{-P_x k_2 t}} \qquad \text{(Eq. 1)}$$

where P_x represents the maximum population and k_2 the second-order reproduction constant. The other symbols are as used already.

For purposes of making calculations and plotting the results, a logarithmic form of the equation, as follows, is more useful:

$$\frac{2.3}{P_x} \log \frac{P_t}{P_x - P_t} \cdot \frac{P_x - P_0}{P_0} = k_2 t \qquad \text{(Eq. 2)}$$

Table II contains the values for the first member of Eq. 2 for the data presented graphically in Fig. 1. It is to be observed that all the values related to a given time are very closely alike. Presumably all populations of cells, normal and trained, in the presence and in the absence of drug, multiplied according to the same law. The agreement of the data with values calculated assuming second-order kinetics may be observed in Fig. 2 where, due to the frequency of the coincidence of points (see Table II), we have plotted only the average of the points for a given time for each type of cell. The slope, or the average value for k_2, amounted to 0.37×10^6 ml./cell/hour.

While some concern may be expressed over the fact that the curve does not meet the origin, a fact which may be due to the lack of control of the pH of the cultures (8) and other factors, there can be little doubt concerning the utility of Eq. 2

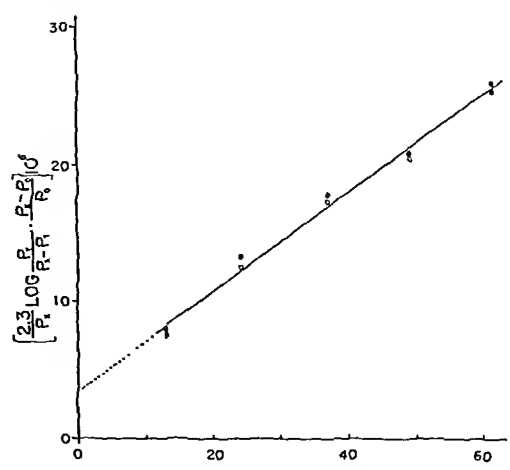

Fig. 2.—The results given in Fig. 1 are plotted according to Eq. 2, with the first member of the equation as ordinate and the time in hours as the abscissa.

TABLE I.—THE VALUES OF SEVERAL CONSTANTS RELATED TO THE RATE OF GROWTH OF *Tetrahymena pyriformis* AND ITS INHIBITION BY URETHANE

Cell Type	Concn. Urethane, Moles/ Liter	k_1 hr.$^{-1}$	P_x $\times 10^3$	P_x/k_1 $\times 10^3$	$k_2 \times 10^{-6}$ ml./cell/ hr.
Normal	0.0	0.417	363	873	0.37[a]
Normal	0.07	0.329	313	950	0.37[a]
Normal	0.08	0.288	273	946	0.37[a]
Normal	0.09	0.259	254	978	0.37[a]
Trained	0.0	0.417	362	873	0.37[a]
Trained	0.07	0.356	326	915	0.37[a]
Trained	0.08	0.336	311	930	0.37[a]
Trained	0.09	0.309	275	890	0.37[a]

[a] Calculated from slope in Fig. 2.

TABLE II.—THE VALUES FOR $k_2 l$ CALCULATED BY USE OF THE FIRST MEMBER OF EQ. 2 FROM THE DATA PRESENTED IN FIG. 1

Cell Type	Concn. Urethane	$k_2 t \times 10^6$ 13 hr.	24 hr.	37 hr.	49 hr.	61 hr.
Normal	0.0	7.4	12.8	17.3	20.2	24.9
Normal	0.07	6.7	11.7	16.3	19.3	25.2
Normal	0.08	6.8	12.9	17.7	21.1	25.7
Normal	0.09	6.3	11.9	17.1	20.8	25.2
Trained	0.0	9.0	13.3	17.5	20.4	24.4
Trained	0.07	7.5	13.3	18.1	20.4	27.3
Trained	0.08	7.4	13.0	16.8	20.2	24.5
Trained	0.09	7.9	13.6	18.7	21.9	27.0

in describing the growth of the cells, both in the presence and in the absence of drug.

In considering the possible biological significance (2, 3, 4) of second-order kinetics, it may be assumed that the rate of cell multiplication is proportional both to the cell population and to some limiting factor. According to Browning, *et al.* (5), the necessary relation can be set forth as follows:

$$\frac{dP_t}{d_t} = k_2 P_t F_t \qquad \text{(Eq. 3)}$$

where F_t is the concentration of the limiter.[1]

While no attempt will be made to consider more precisely the nature of F_t at this time, there is little doubt that this factor is related to the rate of oxygen consumption, which in our studies has been limited either by the supply of oxygen (9, 10, 11) or by the effect of the drug (1). With the aid of even such a sketchy knowledge of F_t, it becomes possible to explain several aspects of the data presented in Fig. 1.

Consider first the curve for the rates of division for both the normal and the trained cells in the absence of drug (Fig. 1). During the first four generations of growth, the rate of solution of oxygen, which is limited by the surface of the culture medium exposed to the atmosphere, exceeds the rate of consumption of oxygen, which in turn is determined by the concurrent cell population. Under these conditions $k_2F_t = k_1$ (drug-free value) and since the medium is saturated with oxygen, the magnitude of F_t is maximal and constant, and growth proceeds exponentially (first order) at a maximum rate. When, in our studies, the cell population exceeds about 20,000–25,000 cells/ml. (between the fourth and fifth generation), the demand for oxygen exceeds its rate of supply and both the rate of respiration and the value of its closely related parameter F_t fall. Under these conditions, second-order growth sets in and continues until the maximum population is reached.

The situation is very little different with the drug-depressed growths. During the early phase of growth in the presence of drug, $k_2F_t = k_1$ (characteristic for each drug concentration) as in the case of drug-free growth, but with the difference that although constant, the value for F_t is not maximal. Indeed, the value for F_t is inversely related to the concentration of the drug due to the respiratory depressing effect of the drug (1). The drug-dependent values of F_t give rise to the values of k_1 characteristic for each drug concentration. Second-order growth sets in, as in the drug-free situation, when the demand for oxygen exceeds the rate of its supply.

According to these views, the linear portion of the curves for the drug-depressed growths should extend to higher population values the greater the concentration of drug, since each increase in concentration would permit a larger population of cells to be associated with a given level of total respiration. Unfortunately, our cell count data were not taken at sufficiently close intervals, during the critical period, to furnish evidence either against or in support of such a proposition. However, it was observed, as might well be expected, that the linear portion of the curve in the drug-free system was extended with increase in the surface to volume ratio of the culture.

The indication that the same growth-limiting factor is affected both by oxygen want and the action of urethane is strongly supported by the fact that growth under both conditions is described by the same equation and characterized by the same second-order reproduction constant. In accepting such a statement, it should be kept in mind that the primary site of action of the drug need not be on electron transport. A primary inhibition of some growth-essential endergonic process would have the same effect.

Although exponential growth accounted for less than 10% of the total increase in cell population, the first-order growth constant appears to have furnished a valid index of the quantitative effects of the drug. As is shown in Table I, the maximum populations attained (P_x) decreased progressively as the concentration of drug increased. The variation in P_x for both types of cells is similar to the variation in k_1. Indeed, the listed values for P_x/k_1 in Table I are nearly constants. The constancy of this relationship indicates that P_x and k_1 are equally good measures of the quantitative effect of urethane. Poole and Hinshelwood (12) observed a similar parallel variation in the mean generation time ($0.693/k_1$) and the maximum population caused by growth-depressing agents. The explanation offered by them may well hold for the action of urethane.

One further point might be made; although the data are limited, the plots for either k_1 or P_x *versus* concentration are linear. This is in conformity with what is frequently observed for narcotic drugs (13).

[1] In the derivation of Eq. 2, F_t is equated to $P_x - P_t$. In a sense, it is a measure of the unborn population since at $t = \alpha$, $F_t = o$ and $P_t = P_x$.

SUMMARY

1. A kinetic analysis of the effect of urethane on the rate of cell division of *Tetrahymena pyriformis* shows that both the drug-free and the drug-inhibited growth are best described by a second-order autocatalytic rate law.

2. The idea is advanced that both urethane and oxygen-want influence the concentration of a rate-limiting factor necessary for growth.

3. Both the first-order reproduction constant (k_1) and the magnitude of the maximum population (P_x) are valid measures of the quantitative effects of urethane.

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A Note on the Synthesis and Pharmacological Activities of a Vinylog of Nikethamide and Some Related Substances*

By HUGH BURFORD†, ALTA R. GAULT, S. T. COKER‡, and W. LEWIS NOBLES

THE RELATIONSHIP of physical and chemical properties of a compound and its vinylogs is well known (1); interest has been manifest by others (2) in attempting to relate the pharmacological actions of a chemical substance and its vinylogs Previously, we (3) have reported on such studies in the areas of antitubercular agents, analgesics, antispasmodics, general antibacterial agents, and thyroxine analogs.

The present study was designed in an effort to extend our previous work with vinylogous compounds to vinylogs of nicotinic acid and its diethylamide

With respect to the pharmacological action of pyridyl-3-acrylic acid, a vinylog of nicotinic acid, both *in vitro* antibacterial screening and an anti-inflammatory test using the granuloma pouch method were conducted with this compound No activity was demonstrated in either test; the LD_{50} of this substance intraperitoneally in mice was determined to be 1,900 mg /Kg [1]

In a preliminary study in our laboratory, the effect of this vinylog of nikethamide was determined by intravenous injection into rats and rabbits With the rats, a closed respiratory system was used; on rabbits, an open system was utilized. In both cases, nikethamide was used as the control standard The vinylog was found to mimic the action of nikethamide on a qualitative scale; the respiratory rate was increased in both types of animals to approximately the same degree with equivalent doses of each drug.

The results of additional tests with this vinylog are summarized as follows[1]: (*a*) CNS: the compound appears to have slight sedative and muscle relaxant properties; no significant behavorial changes in cats with an oral dose of 32 mg /Kg. were noted (*b*) Pharmacodynamic: the profile of this compound in this test indicated it to be a weak ganglionic blocking agent (*c*) Antiscrotonin: in this test, the compound has very little activity

EXPERIMENTAL

Pyridyl-3-acrylic Acid Diethylamide Hydrochloride.—Pyridyl-3-acrylic acid was prepared in 74% yield according to the method of Panizzon (4) This acid was converted to the corresponding acid chloride by heating it at reflux temperature with excess thionyl cnloride. The crude acid chloride was allowed to react with diethylamine on a 1.2 molar basis by placing 10 Gm (0 06 mole) of the acid chloride in 250 ml of dry benzene in a three-neck flask equipped with a rapid stirrer, reflux condenser, and dropping funnel Seven and six-tenths grams (0 12 mole) of diethylamine in dry benzene was added dropwise to the mixture After the addition of diethylamine, the contents of the flask were brought to reflux for fifteen minutes and the solution filtered while hot to remove the diethylamine hydrochloride The filtrate was shaken with a 30% solution of sodium hydroxide The benzene layer was removed and dried over anhydrous sodium carbonate Benzene was removed from the extract and the residue distilled The product distilled in the range of 155–158° at 0 2 mm (Panizzon (4) reports 145° at 0 1 mm) Anhydrous hydrogen chloride was bubbled through a solution of this material dissolved in dry benzene to precipitate the hydrochloride of the desired compound Approximately 12 Gm (62%) of the product, m p 228–230°, was obtained

Anal—Calcd for $C_{12}H_{16}N_2O \cdot HCl$: C, 59 87; H, 7.12 Found: C, 59 53; H, 7 31.

* Received July 3, 1959, from the University of Mississippi, School of Pharmacy, University
Abstracted, in part, from a thesis submitted to the Graduate School of the University of Mississippi by Hugh Burford in August, 1956, in partial fulfillment of the requirements for the M S degree
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[1] Pharmacological results are made possible through the courtesy of Dr George Naimark, White Laboratories, Inc and Dr R M Taylor, Schering Corp

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A Note on the Occurrence of Gramine in *Acer rubrum* L.*

BY IRWIN J. PACHTER

In a previous communication (1) it was reported that the alkaloid gramine occurs in the leaves of the silver maple tree, *Acer saccharinum* L. Since alkaloids had not been reported previously in plants of the maple family, it was of interest to us to check other maples for alkaloid content.

Tests on the leaves and stems of *Acer rubrum* L.[1] showed the presence of traces of alkaloids. The winged fruit[1] gave a stronger test and was therefore subjected to analysis.

From 120 Gm. of dried ground fruit there was isolated 37 mg. of a crystalline alkaloid, m. p. 130–131°. The alkaloid was found to be identical with a synthetic specimen of gramine through mixed melting point determination and comparisons of infrared and ultraviolet spectra. The picrate derivatives of the natural and synthetic specimens were prepared and found to be identical.

EXPERIMENTAL

A 120-Gm. sample of dried fruit of *Acer rubrum* L. was finely ground in a Waring Blendor and allowed to stand under hexane for one day. The hexane solution was removed by filtration. It gave a negative test for alkaloids and was discarded. The marc was then stirred under reflux with 95% ethanol for three hours and filtered. Extraction with ethanol was repeated five times. The alcoholic solutions were combined and evaporated to dryness. The residue was dissolved by shaking with 200 ml. of ethyl acetate and 200 ml. of 2% aqueous ammonia. Twenty grams of sodium chloride was added to hasten separation of the organic and aqueous phases. The ethyl acetate layer was shaken with three 100-ml. portions of 2% hydrochloric acid. The acid solutions were combined, made strongly basic with concentrated ammonia, and extracted with three 100-ml. portions of ether. The ethereal solutions were combined, dried over magnesium sulfate, and evaporated to dryness. The residue crystallized. It was recrystallized from benzene to yield 37 mg. of crystalline gramine, m. p. 130–131°. Upon admixture with an authentic sample of gramine, m. p. 131–132°, there was no depression of melting point.

The alkaloid formed a picrate, m. p. 140–141°, which did not depress the melting point of authentic gramine picrate.

* Received September 4, 1959 from the Research and Development Division, Smith Kline and French Laboratories, Philadelphia 1, Pa.
The author is grateful to Mr. Herman Farber for laboratory assistance.

[1] The samaras were collected in February and the leaves and stems in April in the Tallahassee, Florida, area by Professor R. K. Godfrey of the Florida State University. Herbarium specimens, Nos. 58,200 and 58,323, have been deposited at the herbarium of the Florida State University.

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Book Notices

Symposium on Nuclear Sex. Edited by D. Robertson Smith and William M. Davidson. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1958 xvii + 188 pp. 14 x 21.5 cm. Price $3.50.

The papers and discussions at the symposium are introduced with a reference to the classical paper of Polani, Hunter, and Lennox which showed that some women with what was then called ovarian agenesis seemed to be genetic males. The papers are grouped under the headings: Cytological and genetic aspects of nuclear sex, Application to the study of intersex and related states, and Application to the study of tumors.

Clinical Evaluation of New Drugs. Edited by S. O. Waife and Alvin P. Shapiro. Paul B. Hoeber, Inc., Medical Book Dept. of Harper & Brothers. 49 East 33rd St., New York 16, N. Y., 1959. x + 223 pp. 15.5 x 23.5 cm. Price $7.50.

The methods and standards by which new therapeutic agents can be evaluated are presented. Each of the 14 chapters in the book has been written by an author experienced in the area covered. The steps leading from the inception of a drug to its application are adequately discussed, and criteria are presented by which the medical practitioner can judge critically how thoroughly a new agent has been investigated. The ethics of human experimentation, placebos, evaluation of subjective responses, statistical problems, and funds for research are some of the subjects that have been covered. On clinical trials in practice, chapters are devoted to infectious and gastrointestinal diseases and to nutritional and metabolic, cardiovascular, and psychiatric disorders. This book should be of special importance to all who have an interest in new drugs.

Mechanisms of Hypersensitivity. Henry Ford Hospital International Symposium. Edited by Joseph H. Shaffer, Gerald A. LoGrippo, and Merrill W. Chase. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1959. xx + 754 pp. Price $18.50.

Sixty-five specialists working in the fields of allergy, dermatology, immunology, and allied sciences have contributed to this book. Their latest findings on immunologic processes as they pertain to problems of hypersensitivity are presented. The individual topics cover and integrate a large number of subjects, such as factors that condition the individual to undergo sensitization, the diverse types of antibodies, the apparent molecular heterogeneity within the recognized types and newer methods of detecting them, new aspects of the physiological control of histamine in allergy, and evidence for a role of serotonin. Many other topics related to the mechanisms of hypersensitivity are discussed, and the book should serve adequately as a standard reference on the subject.

Scientific Edition

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Some Aspects of the Central Nervous System Activity of Urazole Derivatives II*

By C. L. MITCHELL†, H. H. KEASLING, and C. W. HIRSCHLER

The neurotoxicity, antielectroshock, anti-Metrazol, and antistrychnine activities of another series of urazole compounds have been determined in mice. Depending upon the nature of the substituted nitrogen groups, the members of this series, as with the previous compounds, were found to possess antielectroshock, anti-Metrazol, antistrychnine, convulsant, and depressant type actions. In general, the urazole compounds are most effective against maximal Metrazol seizures.

THE SIMILARITY of the urazole and hydantoin nucleus prompted an investigation of the C N. S. activity of a series of substituted urazoles One group of these derivatives has been reported previously (1) The compounds were relatively short acting and nontoxic. In general, they were found to be most effective against maximal Metrazol seizures. Variations in the substituted nitrogen groups yielded compounds possessing anticonvulsant, convulsant, and depressant type actions.

This report concerns the C. N. S. activity of another series of urazole derivatives and some observations concerning the structure-activity relationship of the C. N. S actions of the entire series

EXPERIMENTAL

Adult male albino mice obtained from the Arthur Sutter Farms (Rockland strain), weighing 20 to 25 Gm., were maintained on Purina laboratory chow and allowed free access to food and water except during the test period The mice were used for one experiment only The test procedures employed were: (*a*) the supramaximal electroshock seizure technique (M. E. S), of Toman, *et al.* (2), in which alternating current, 50 ma. of 0.2 second duration was delivered through Spiegel corneal electrodes (3) from an electroshock apparatus constructed according to the design of Woodbury and Davenport (4); (*b*) the maximal Metrazol seizure test (M. M. S), of Goodman, *et al* (5), in which Metrazol,[1] 38 mg./Kg , was injected intravenously. The end points for both tests were the abolition of the tonic extensor components of the seizure pattern; and (*c*) ability to prevent death from strychnine, in which 2.5 mg /Kg. of strychnine was injected intraperitoneally (6).

All compounds were suspended in 10% acacia for oral administration. The convulsant stimulus was delivered thirty minutes following urazole administration, with the exception of UR Nos. 103 and 104. The convulsant stimulus for the latter two compounds was delivered one hour (UR 103) and one and one-half hours (UR 104) following drug administration Those compounds exhibiting protection at 1 Gm /Kg. from electroshock and/or Metrazol were then administered in dosages of 300, 100, and 30 mg /Kg until less than 50% of the animals were protected

Acute neurotoxicity (N T) was determined by the method of Swinyard, *et al* (7) The following end points were used: (*a*) positional sense test, (*b*) righting test, (*c*) gait and stance test, (*d*) muscle tone test, and (*e*) equilibrium test. Neurotoxicity was recorded when an alteration in response to any one of the above procedures was observed. The mice were examined at fifteen-minute intervals for at least one hour, and thereafter until a decrease in the number of mice exhibiting neurological symptoms was seen

The 50% neurotoxic dose (TD_{50}) and the 50% effective dose (ED_{50}) for UR 110 and UR 104 were determined at thirty minutes and one and one-half

* Received June 27, 1959, from the College of Medicine, State University of Iowa, Iowa City

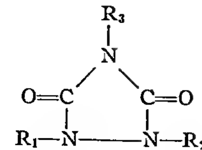
† Smith, Kline and French Fellow in Pharmacology

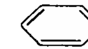
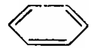
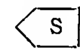
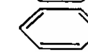
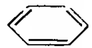
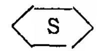
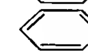
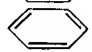
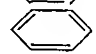
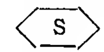
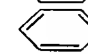
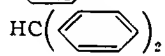
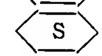
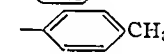
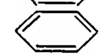
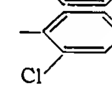
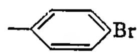
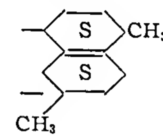
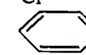
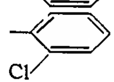
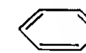
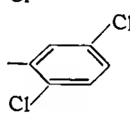
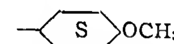
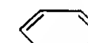
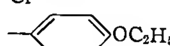
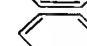
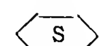
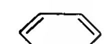
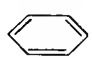
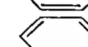
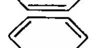
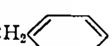
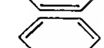
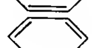
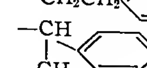
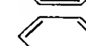
The urazole compounds were kindly supplied by and the work was supported in part by the Knoll Pharmaceutical Co , Orange, N J , and in part by U S P H S Grant B-1079

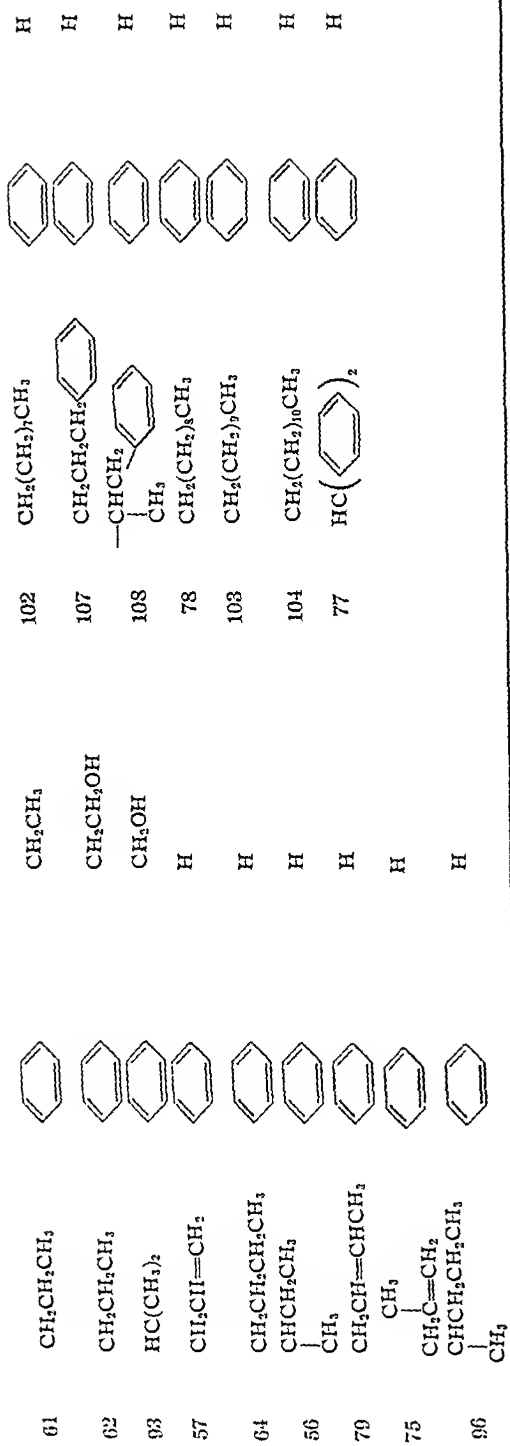
The material contained in this report is to be submitted to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Ph D

[1] The Metrazol was kindly supplied through the courtesy of Dr R O Hauck, Knoll Pharmaceutical Co

TABLE I.—STRUCTURES OF URAZOLE DERIVATIVES



UR No.	R$_1$	R$_2$	R$_3$	UR No.	R$_1$	R$_2$	R$_2$
100	H		CH_2CH_3	69	$HC(CH_2CH_3)_2$		H
109	H		$CH_2CH_2CH_3$	63			H
111	H			94	$CH_2(CH_2)_4CH_3$		H
105	H			92			CH_2OH
54	H		H	85			H
80	CH_3		H	88			H
81	CH_3		H	66			H
90	CH_3		H	70			H
91	CH_3		H	72			H
				71	$CH_2(CH_2)_5CH_3$		H
89	CH_3		H	67	$HC(CH_2CH_2CH_3)_2$		H
110	CH_2CH_3	CH_2CH_3	H	65			H
59	CH_2CH_3		CH_3	101	$CH_2(CH_2)_6CH_3$		H
58	CH_2CH_3		CH_2CH_3	73	CH_2CH_2		H
60	CH_2CH_3		CH_3	83			H



hours, respectively. Similar tests for trimethadione,[2] phenobarbital, and diphenylhydantoin[3] were determined at one and one-half hours, two hours, and three hours, respectively. These time intervals represent the approximate time of peak effectiveness for each drug, as determined in our laboratories utilizing the M. E. S. technique. The time of peak effect for the standard anticonvulsants is in agreement with that reported by Swinyard, *et al.* (7), and Chen, *et al* (8).

The ED_{50} in the Metrazol seizure threshold test (Met.) of Swinyard (7), was also determined for the above compounds. In this test, Metrazol, 85 mg./Kg. was injected subcutaneously; the end point was the abolition of clonic spasms. In the Met. test, the Metrazol was injected subcutaneously ten minutes prior to the time of peak effect and the mice were observed for a period of thirty minutes.

The computations for fitting the probit-log dose regression line, the determination of the TD_{50}, ED_{50}, protective index (P.I.), and the 95% confidence interval were done according to the methods described by Finney (9). In determining the ED_{50} at least three dose levels, yielding between 10 and 90% effect were used. At least 10 mice were utilized in determining each point.

The time of peak anticonvulsant effect and duration of action of one compound (UR 104) was determined by the M. M. S. test. A dose of 100 mg./Kg. was administered to five groups of 10 animals. The respective groups of mice were then injected with Metrazol at thirty, sixty, ninety, one hundred and twenty, and one hundred and eighty minutes.

The sleeping time produced by two compounds (UR 75 and 96) was compared with hexobarbital Sleeping time was recorded as the time interval in which the righting reflex of the mice was absent The effect of five compounds (UR 73, 83, 107, 108, and 109) upon the sleeping time of hexobarbital was also determined. The convulsant compounds were administered orally, two minutes prior to the i p injection of hexobarbital. The 95% confidence intervals were calculated according to the method described by Snedecor (10).

RESULTS

The structures of the urazole derivatives studied are detailed in Table I. UR Nos. 109, 73, 83, 107, and 108, which were convulsants, produced death in 50% or more of the mice at 1 Gm./Kg. Less than 50% of the mice were killed at 1 Gm./Kg. with the following compounds: UR Nos. 62, 93, 94, 111, 59, 58, and 88. The remaining compounds were relatively nontoxic at 1 Gm./Kg. (i. e., no mice were killed). The predominant neurotoxic effect was ataxia.

The anticonvulsant activities are detailed in Table II. Data for phenobarbital, diphenylhydantoin, and trimethadione have been reported previously (1) and are in general agreement with those of Swinyard, *et al.* (7), Goodman, *et al.* (5), and Chen, *et al.* (8). As with the previously studied materials (1), the present urazole compounds possess relatively good anti-M. M. S. activity, and they are

[2] The trimethadione was kindly supplied through the courtesy of Dr. R. K. Richards, Abbott Laboratories.
[3] The diphenylhydantoin was kindly supplied through the courtesy of Dr. Graham Chen, Parke, Davis and Co.

ineffective in the M. E. S test in non-neurotoxic doses.

Neurotoxicity tests indicated that all active members of this series were rapidly absorbed from the intestinal tract and with the exception of UR Nos 103, 104, and 110, had a relatively short duration of action All of the urazole derivatives reported previously (1) appeared to have a short duration of action Time duration studies on UR 104, 100 mg /Kg (Fig. 1), indicate that this compound has a peak effect ninety minutes after oral administration Some anticonvulsant activity is still present three hours after administration A similar study of UR 110 revealed a peak effect at thirty minutes

The TD_{50} and the ED_{50}'s in the M M. S , Met , and M E S tests for two of the more active members of the series (UR 104 and UR 110) were determined A comparison of these data with those of trimethadione, phenobarbital, and diphenylhydantoin are presented in Table III From the data presented, it would appear that UR 104 and UR 110 are relatively specific antagonists of Metrazol.

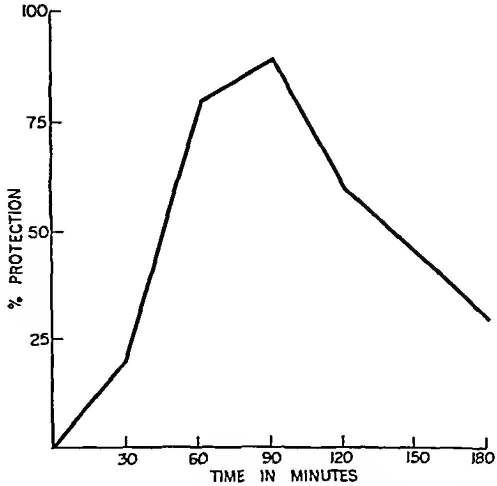


Fig 1 —Duration of action of UR 104, 100 mg /Kg

TABLE II —ANTICONVULSANT ACTIVITY OF URAZOLES[a b,c]

UR No	Test	1,000	300	100	30	UR No	1 000	300	100	30
111	NT[d]	15/20					20/20	20/20	5/20	0/10
	MES[e]	0/10				96	10/10	10/10	0/10	..
	MMS[f]	0/10					10/10	9/9	6/10	2/9
80	NT	20/20	20/20	0/10			20/20	5/20	0/10	..
	MES	10/10	1/10			92	7/9	0/9		.
	MMS	8/8	10/10	2/8			9/9	8/9	0/10	
81	NT	16/16	9/20				0/20			.
	MES	4/8	1/10			85	0/8			
	MMS	8/8	3/9				8/8	5/10	1/10	
90	NT	20/20	10/10	0/10			20/20	11/20	0/10	
	MES	3/9				88	5/8	2/10		
	MMS	9/9	7/7	4/10			8/8	7/9	6/10	2/10
91	NT	20/20	0/10	0/10			12/20	4/10	2/10	
	MES	8/8	0 10			66	8/9	0/10		
	MMS	7/7	9/10	1/10			5/6	5/8	4/10	
89	NT	0/20					0/20			
	MES	0/9				70	0/10			
	MMS	3/9					7/10	0/9		
110	NT	6/10	1/20	0/10			6/20			
	MES	0/10				72	0/10			
	MMS	10/10	8/10	6/10	2/10		8/9	1/10		
59	NT	20/20	0/20				20/20	7/18	0/10	
	MES	9/9	0/10			71	6/10	0/8		
	MMS	8/8	4/9				9/10	4/8	0/10	.
58	NT	20/20	0/10				1/20	0/10		
	MES	1/10				67	3/9			..
	MMS	5/10	1/10				7/8	3/10		.
60	NT	20/20	0/10				8/20	2/10		.
	MES	3/10				65	0/10			
	MMS	7/7	1/8				9/9	3/8		..
61	NT	11/17	0/10				20/20	1/10	0/10	..
	MES	1/10				78	0/10			
	MMS	5/7	2/9				9/9	8/9	5/10	1/10
62	NT	20/20	7/20				20/20	0/10	0/10	..
	MES	10/10	4/10			103	2/10			..
	MMS	9/9	4/9				10/10	9/10	2/10	..
93	NT	20/20	17/20	0/10			20/20	10/10	0/10	..
	MES	8/9	3/10			104	2/10			..
	MMS	10/10	9/9	4/9			10/10	10/10	8/10	2/10
75	NT	19/19	10/10	0/10						
	MES	8/8	2/10							
	MMS	8/8	10/10	7/10	1/7					

[a] UR Nos 100, 105, 54, and 77 inactive at 1 Gm /Kg
[b] UR Nos 109, 73 83 107, and 108 are convulsant (see text)
[c] UR Nos 57, 64, 56, 79, 75, 96, 69 63, 94, 101, and 102 stimulate, then depress at 1 Gm /Kg (see text).
[d] NT = Neurotoxicity (see text)
[e] MES = Supramaximal electroshock seizure technique (see text)
[f] MMS = Maximal Metrazol seizure test (see text)

TABLE III.—TOXICITY, ANTI-M. M. S. POTENCY, ANTI-MET. POTENCY AND ANTI-M. E. S. POTENCY FOR UR 104, 110, TRIMETHADIONE, PHENOBARBITAL, AND DIPHENYLHYDANTOIN[a]

Drug	Time,[b] Min.	Neurotoxicity, TD_{50}, mg./Kg.	M. M. S. Potency, ED_{50}, mg./Kg.	Met. Potency, ED_{50}, mg./Kg.	M. E. S. Potency, ED_{50}, mg./Kg
UR 104	90	204 (55–684)	55 (35–75)	88 (46–131)	820 (638–989)
UR 110	30	875 (702–1099)	80 (30–130)	273 (169–422)	>1000
Trimethadione	90	975 (760–1390)	236 (196–281)	421 (275–556)	1082 (923–1262)
Phenobarbital sodium	120	60 (47–77)	9 (6–12)	27 (17–36)	21 (17–25)
Diphenylhydantoin sodium	180	100 (56–184)	11 (9–12)	Ineffective in doses up to 100 mg./Kg.	14 (11–17)

[a] Values in parentheses represent the 95% confidence interval.
[b] Time elapsed after administration of drug before Metrazol was injected.

Compounds No. 73, 83, 107, 108, and 109 produced convulsions at 1 Gm./Kg. With the exception of UR 109, the convulsions were primarily clonic in nature. Tonic flexion was the most pronounced effect produced by UR 109. All of the compounds produced convulsions within ten minutes. UR Nos. 73, 83, 107, and 108, at 1 Gm./Kg. produced death in all of the animals within one hour after administration. At 300 mg./Kg., UR 73 and UR 83 produced periodic mild clonus which lasted for approximately one and one-half hours. Mice receiving these two compounds in a dose of 100 mg./Kg. appeared hyperactive. UR 107, 300 mg./Kg. produced death in three out of ten animals, mild stimulation was noted from 100 mg./Kg. UR 108, 300 mg./Kg. produced death in all animals. At 100 mg./Kg., hyperactivity was noted. UR 109, 1 Gm./Kg., produced death in four out of 10 animals within one and one-half hours after administration. No effect was seen from this compound at 300 mg./Kg. The effect of these compounds on the sleeping time of mice receiving 150 mg./Kg. of hexobarbital administered i. p. is detailed in Table IV. UR 107, 100 mg./Kg., appeared to prolong the sleeping time of mice receiving hexobarbital. The other compounds, in the dose studied, had no apparent effect.

Compounds No. 57, 64, 56, 79, 75, 69, 63, 94, 101, and 102, 1 Gm./Kg., caused initial stimulation which was followed by depression. However, only UR 75 and UR 96 induced a loss of righting reflex at 400 mg./Kg. Table V shows the mean sleeping time of UR 75, and UR 96, 400 mg./Kg., as compared to a similar dose of hexobarbital.

All compounds (except UR 100 and UR 105) were tested at a dose of 1 Gm./Kg. for their ability to protect against death produced by i. p. injection of 2.5 mg./Kg. of strychnine. Only UR Nos. 80, 81, 90, and 89 provided significant protection. The activity of these compounds in comparison with 250 mg /Kg. of Zoxazolamine[4] and 500 mg./Kg. of mephenesin is detailed in Table VI. It would appear that UR 80 and UR 81, 250 mg./Kg., are comparable to Zoxazolamine.

TABLE IV.—EFFECT OF UR 73, UR 83, UR 107, UR 108, AND UR 109 UPON THE MEAN SLEEPING TIME OF HEXOBARBITAL, 150 MG./KG. I. P. IN THE MOUSE

Hexobarbital, 150 mg./Kg. i. p. With-	Mean Sleeping Time, Min.	95% Confidence Interval
Nothing	51	36–65
UR 73 100 mg./Kg. (oral)	63	46–80
UR 83 100 mg./Kg. (oral)	71	54–88
UR 107 100 mg./Kg. (oral)	138	77–200
UR 108 100 mg./Kg. (oral)	83	56–116
UR 109 500 mg./Kg. (oral)	68	55–81

TABLE V.—MEAN SLEEPING TIME OF UR 75, UR 96, AND HEXOBARBITAL, 400 MG./KG., ORALLY

Drug	Mean Sleeping Time, Min.	95% Confidence Interval
UR 75	19	1–36
UR 96	22	3–40
Hexobarbital	41	33–48

TABLE VI.—ABILITY TO PREVENT DEATH FROM I. P INJECTION OF 2.5 MG./KG. OF STRYCHNINE

Drug	Oral Dose	Time,[a] Min.	No. of Animals Surviving/ No. of Animals Tested
UR 80	250 mg./Kg.	30	4/10
UR 81	250 mg./Kg.	30	6/10
UR 90	1 Gm./Kg.	30	3/10
UR 89	1 Gm./Kg.	30	6/10
Zoxazolamine	250 mg./Kg.	60	4/10
Mephenesin	500 mg./Kg.	5	3/10

[a] Time elapsed after administration of drug before strychnine was injected.

DISCUSSION

In our previous report (1), factors validating a discussion of the structure-activity relationship of the urazole derivatives were enumerated and the following observations were made: (*a*) at least one phenyl group on the adjacent nitrogen atom (R_1 or R_2, Table 1) is essential for anticonvulsant activity; (*b*) if R_1 or R_2 is phenyl, all other substituents except hydrogen on the adjacent nitrogen can confer anticonvulsant activity; and (*c*) the R_4 substituent must be small or relatively polar for maximal anticonvulsant activity.

The results obtained in the present series indicate that while a phenyl group on one of the adjacent ni-

[4] The Zoxazolamine was kindly supplied through th courtesy of Dr. Charles F. Kade, Jr., McNeil Laboratorie Inc.

trogen atoms is sufficient for anticonvulsant activity, it is not necessary, since UR 110 (1,2-diethyl urazole) was one of the more active compounds in this series. This was the only compound, however, which possessed anticonvulsant activity and did not contain a phenyl group on one of the adjacent nitrogen atoms

In this series, when R_1 or R_2 is phenyl, most of the groups except hydrogen do confer anticonvulsant activity (compare UR 81, 60, 61, 93, 57, 64, 56, 79, 75, 96, 69, 63, 94, 92, 66, 72, 71, 67, 65, 101, 102, 78, 103, and 104, with UR 100, 109, 111, and 105) However five compounds (UR 73, 83, 107, 108, and 77) did not possess anticonvulsant activity All of these compounds, except UR 77 (which was inactive at 1 Gm /Kg) were convulsants

Although the number of comparisons in this series is limited, the results would seem to substantiate the conclusion that the R_3 substituent must be small or relatively polar for maximal anticonvulsant activity (compare UR Nos 59 and 58, UR 62 and 61)

UR Nos. 92, 85, 88, 66, 70, and 72 are derivatives of one of the more active anticonvulsant urazole compounds reported previously (UR 20, 1-phenyl-2-cyclohexyl urazole) (1) Compounds No 92, 85, 70, and 72 are less active than UR 20 UR 88 and UR 66 are approximately equal to UR 20 in anticonvulsant activity However, UR 88 appears to be more neurotoxic than UR 20.

UR Nos 80, 81, 90, 91, and 89 are derivatives of the only compound (UR 8, 1 phenyl-2-methyl urazole) reported previously (1) which possessed significant antistrychnine activity These compounds were the only members of this series which possessed antistrychnine activity. UR 80 and UR 81 are more active than UR 8, while UR 90 and UR 89 are approximately equal in activity to UR 8 UR 91, 1 Gm /Kg., was devoid of antistrychnine activity

In this series, as in the urazole derivatives reported previously, a variety of actions was noted. The anti-Metrazol activity, however, appears to be one of the more striking properties of the urazoles.

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The Physical Chemical Evidence for Aspirin Anhydride as a Superior Form for the Oral Administration of Aspirin*

By EDWARD R. GARRETT

Physical chemical and kinetic investigations show that aspirin anhydride is a source of aspirin and that it should not produce digestive upset because of salicylic acid production or undissolved aspirin that may adhere to the gastric mucosa and irritate. On oral ingestion, aspirin anhydride should give higher acetylsalicylate blood levels than aspirin alone due to the latter's pH-dependent intestinal absorption. Complete equations are presented for aspirin anhydride's homogeneous and heterogeneous hydrolysis and solubility and the specific equations for physiological conditions have been derived. Procedures have been developed for the assay of aspirin anhydride and all probable derivatives.

The mechanism of the analgetic action of acetylsalicylic acid, aspirin, has been subject to much discussion The possible side effects are of widespread interest Without introducing an excessive review or critique of the literature, several rational opinions based on experimental evidence may be stated

The onset and duration of aspirin concentration in the blood can be correlated with analgetic relief and this action may be expressed mainly by the unhydrolyzed acetylated fraction in the plasma (1, 2) Aspirin can cause gastric irritation, nausea, and even gastrointestinal bleeding in a considerable number of people (3–6). Disturbances from acetylsalicylic acid are reported to be less serious than from salicylic acid (7), and annoyances of the former have been ascribed to the immediate area surrounding particles (4). Here, irritation could be ascribed to the acid function. There is evidence that soluble material is less irritating (6).

It rationally follows that if a form of "aspirin" could produce equivalent aspirin blood levels and less salicylic acid than acetylsalicylic acid, the

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net result could be less gastric irritation without lessened analgetic activity. Further, if this "aspirin" were nonacidic and produced higher aspirin blood levels, it might even be found to be superior to acetylsalicylic acid as the optimum form for administration of this drug.

This paper presents and considers the physicochemical and kinetic investigations on aspirin anhydride which permit consideration of it as a superior "aspirin."

EXPERIMENTAL

When the aspirin or salicylic acid impurities of aspirin anhydride (AA) were to be determined quantitatively simultaneously with the rate study for the determination of its heterogeneous hydrolysis, 100 mg. AA were accurately weighed into a 15 × 45 mm. glass vial (Kimble Opticlear) with a press fit polyethylene cap. Four milliliters of 0.1000 N HCl were pipetted into the vial. The HCl had been previously maintained at the temperature of the study. The vials were strapped horizontally on a bar suspended vertically into a constant temperature bath. The upper portion of the bar was clamped on the horizontal arm of a Burrell wrist-action shaker, the lower end of the bath was free. The net action of the vials was elliptical and gave excellent agitation of the dispersed AA in the liquid. Quantitative weighing of AA was not necessary when only solubility or heterogeneous rate were to be determined.

The AA was filtered from the solution by a sintered glass filter (medium).

In run 1 (see Table I and Fig. 1) both filtrate and residue were assayed spectrophotometrically for AA content by the following procedure. Two ml. of filtrate, or 3.5 mg. residue, were made up to 25 ml. with dioxane, i. e., solution A. To 10 ml. of solution A were added 10 ml. 0.250 M HCl and the final volume, B, was made up to 25 ml. with dioxane. Also, to 10 ml. of solution A were added 10 ml. 0.100 M sodium acetate and the final volume, B', was made up to 25 ml. with dioxane. Spectra of both solutions, B and B', were run immediately on the Cary spectrophotometer against the appropriate blanks. Typical Cary model 11 curves for aspirin anhydride, salicylic acid, and aspirin are given in Fig. 2.

No significant change was observed in the spectra of aspirin anhydride within ten minutes when in such solutions.

Since the background absorbance of the aqueous dioxane changes with time, especially when in contact with HCl, the spectrophotometric blanks were prepared with the same dioxane at the same time of sample preparation. The dioxane used was distilled on the same day of use and the initial fractions were discarded. The remaining dioxane was purged with nitrogen and not permitted to stay in contact with any aqueous solvent for any great interval.

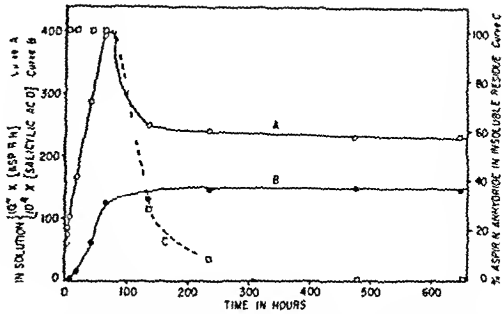


Fig. 1.—Effect of time of heterogeneous hydrolysis on the concentrations of aspirin anhydride and its hydrolytic derivatives. The aspirin anhydride, 100 mg., was equilibrated in 4 ml. 0.1 N HCl at 25.8°. Curve A represents the molar concentration of aspirin and curve B that of salicylic acid in solution. Curve C is the % aspirin anhydride in the undissolved portion.

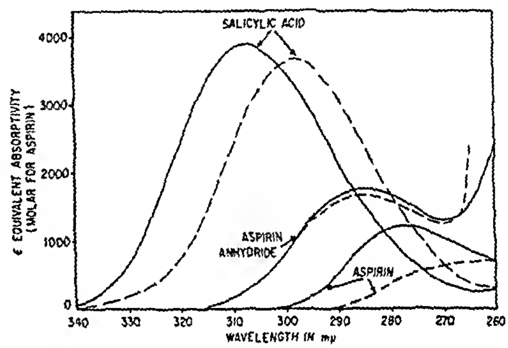


Fig. 2.—Absorptivities of aspirin anhydride and hydrolytic derivatives in 60% (by vol.) dioxane. The solid curves are spectra in 0.1 N HCl. The dashed curves are spectra in 0.1N sodium acetate.

TABLE I.—ESTIMATION OF SOLUBILITY OF ASPIRIN ANHYDRIDE [AA]$_s$ AT VARIOUS TEMPERATURES IN AQUEOUS SOLUTION (100 MG. IN 4 ML. 0.1000 N HCl)

Run	Compd.	°C	10^6k (sec.$^{-1}$) In 10% Dioxane[a]	10^6k (sec.$^{-1}$) In 0% Dioxane[b]	Slope $10^7d\{[asp] + [sal]\}/dt$ (M/L./sec.)	Intercept $10^4[asp]_0$	Intercept $10^4[AA]_s$
1	1	25.8	8.03	20.0	1.85	67	0.457
2	2	25.7	8.02	20.0	1.83	0	0.463
3	3	30.3	9.65	24.1	2.50	4	0.520
4	2	40.1	16.0	40.0	8.81	0	1.101
5	2	50.4	25.4	63.5	24.9	0	1.961

[a] The variation of k in 0.1000 N HCl 10% dioxane had been previously determined as a function of temperature (8), where $\log k = 1960/T + 3.46$ and where $T = 273 + °C$.
[b] The effect of per cent dioxane on k at 25° is linear in the range 0–10% dioxane, i. e., $k = -0.0436$ [per cent dioxane] + 0.3 (8). It was assumed that the effect of decreasing dioxane content on k was the same for all temperatures so that $k^{25.7°}_{0\%\ diox.} / k^{25.7°}_{10\%\ diox.} \times k^{T_s}_{10\%\ diox.} = k^{T_s}_{0\%\ diox.}$, where the former quotient is 20/8.

Several three-component assays gave no indication of any significant amount of AA in the filtrate Thus the aspirin and salicylic acid content of the filtrate was calculated from the absorbances, A, at 307 5 mμ and 277 5 mμ in the HCl dioxane solutions of the Cary model 11 using the following equation

$$[\text{aspirin}] = (A_{277\ 5} - \epsilon_{sal}^{277\ 5}\,[\text{sal}])/\epsilon_{asp}^{277\ 5} \qquad \text{(Eq 1)}$$

where $\epsilon_{sal}^{277\ 5} = 805$, $\epsilon_{asp}^{277\ 5} = 1{,}210$, and $[\text{sal}] = A_{307\ 5}/\epsilon_{sal}^{307\ 5}$, where $\epsilon_{sal}^{307\ 5} = 3{,}890$

The aspirin anhydride content (AA) of the residue was calculated from the absorbances, A, in the HCl-dioxane solutions on the Cary model 14 using the following equation

$$[\text{AA}] = \frac{\epsilon_{asp}^{270}\,[A_{292} - \epsilon_{sal}^{292}A_{320}/\epsilon_{sal}^{320}] - \epsilon_{asp}^{292}\,[A_{276} - \epsilon_{sal}^{276}A_{320}/\epsilon_{sal}^{320}]}{\epsilon_{AA}^{292}\epsilon_{asp}^{270} - \epsilon_{asp}^{292}\epsilon_{AA}^{276}} \qquad \text{(Eq 2)}$$

where $\epsilon_{asp}^{270} = 1{,}200$, $\epsilon_{sal}^{292} = 2{,}700$, $\epsilon_{sal}^{320} = 1{,}898$, $\epsilon_{asp}^{292} = 250$ $\epsilon_{AA}^{292} = 1{,}490$, and $\epsilon_{AA}^{276} = 1{,}540$, where ϵ^{λ}_{AA} is the *equivalent absorptivity = 0 5 × molar absorptivity* for aspirin anhydride

The composition of the filtrate in all other runs at 25 7, 30 3, 40 1, and 50 4° was determined on the basis of negligible anhydride content and use of the following procedure Two milliliters of filtrate were diluted to 25, 50, or 100 ml with 0 1000 N HCl, the amount dependent on the magnitude of the absorbance of the dissolved aspirin and salicylic acid Readings were taken on the Cary model 11 at 277 5 and 394 mμ and the amounts of aspirin, [asp], and salicylic acid, [sal], were calculated from the following equations

$$[\text{sal}] = A_{304}/\epsilon_{sal}^{304} \qquad \text{(Eq 3)}$$

where $\epsilon_{sal}^{304} = 3{,}524$, and

$$[\text{asp}] = (A_{277\ 5} - \epsilon_{sal}^{277\ 5}\,[\text{sal}])/\epsilon_{asp}^{277\ 5} \qquad \text{(Eq 4)}$$

where $\epsilon_{sal}^{277\ 5} = 1{,}025$ and $\epsilon_{asp}^{277\ 5} = 1{,}128$

RESULTS

Since aspirin anhydride in homogeneous aqueous solution undergoes pseudo first-order hydrolysis to aspirin which may subsequently hydrolyze to salicylic acid (8, 9), the hydrolysis of aspirin anhydride in saturated solution should be apparent zero order (10) and

$$d[\text{AA}]/dt = -k[\text{AA}]_s = \text{constant} \qquad \text{(Eq 5)}$$

where $d[AA]/dt$ is in moles of aspirin anhydride hydrolyzed to aspirin per liter per second, k is the pseudo first order rate constant in homogeneous solution (sec $^{-1}$) and $[\text{AA}]_s$ is the solubility of anhydride in moles per liter.

Since

$$-d[\text{AA}]/dt = (1/2)d\{[\text{asp}] + [\text{sal}]\}/dt \qquad \text{(Eq 6)}$$

the knowledge of the rate of appearance of aspirin and salicylic acid will permit the evaluation of $k[\text{AA}]_s$ and knowledge of k will permit estimation of the solubility of $[\text{AA}]_s$ in saturated solution It follows that the plots of [asp] + [sal] *vs* time at a constant temperature and pH should be linear Such plots are given in Figs 3 and 4 for the data

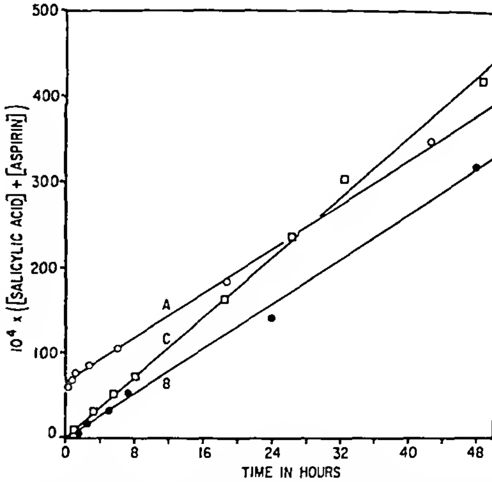


Fig 3—Effect of aspirin contaminant of aspirin anhydride on plot of hydrolytic products as a function of time for 100 mg per 4 ml 0 1 N HCl Curve A is for compound 1 at 25 8°, curve B is for compound 2 at 25 7°, and curve C is for compound 3 at 30 3° (see Table I)

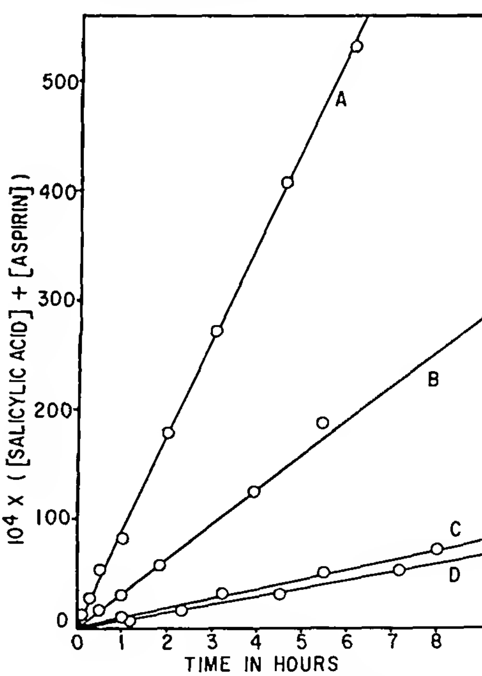


Fig 4—Plot of concentrations of hydrolytic products of aspirin anhydride in saturated 0 1 N HCl solution as a function of time Curve A is at 50 4°, curve B is at 40 1°, curve C is at 30 3°, and curve D is at 25 7°

The slopes so determined along with the k values from homogeneous hydrolysis permit estimation of the solubility, $[\text{AA}]_s$, as given in Table I

The intercept provides an independent estimate of the amount of aspirin contaminant In this regard, this method can serve as an analytical procedure The compound 1 has 4 8% aspirin, the compound 2 has 0 0% aspirin, and the compound

3 has 0 3% aspirin if the three, six, and eight hours alone are used (see Fig 3)

The dependence of anhydride solubility on temperature may be expressed by:

$$[AA]_s = P_s e^{-\Delta H_s/RT} \qquad \text{(Eq. 7)}$$

where ΔH_s is the heat of solution in Kcal./mole, R is the gas constant (1 987 calories degree^{-1} mole^{-1}) and T is the absolute temperature Similarly, the dependence of heterogeneous hydrolysis rate may be expressed by:

$$\begin{aligned} d[AA]/dt &= k[AA]_s = Pe^{-\Delta H_T/RT} \\ &= P_a e^{-\Delta H_a/RT}\, P_s e^{-\Delta H_s/RT} \\ &= (P_a P_s) e^{-(\Delta H_a + \Delta H_s)/RT} \qquad \text{(Eq. 8)} \end{aligned}$$

where ΔH_a is the heat of activation for the homogeneous hydrolysis of aspirin anhydride in 0 1000 N HCl.

The logarithmic forms of Eqs 7 and 8 are plotted in Fig 5 as based on the data of Table I. The results are tabulated in Table II.

Thus, the heat of solution (ΔH_s) for aspirin anhydride is 11 2 Kcal./mole where the apparent heat of activation (ΔH_T) for the pseudo-zero order heterogeneous hydrolysis is 20 5 Kcal /mole The heat of activation for the homogeneous hydrolysis of aspirin anhydride is $\Delta H_a = \Delta H_T - \Delta H_s = 9\ 3$ Kcal /mole

It has been previously shown in studies on homogeneous hydrolysis that the ΔH_a in 0 1 N HCl, 10% dioxane, was 9 9 Kcal /mole (8). The above calculation is confirmatory.

TABLE II.—DATA ON TEMPERATURE DEPENDENCE OF ASPIRIN ANHYDRIDE SOLUBILITY, $[AA]_s$ IN M/L., AND RATE OF ANHYDRIDE HYDROLYSIS, $-d[AA]/dt$ IN M/L /SEC , IN 0.1 N HCl

Dependent	S^a	log P	ΔH (Kcal / mole)
$[AA]_s$	2,445	3 845	11 2
$d[AA]/dt$	4,475	7 927	20 5
k	2,030[b]	4 082[b]	9 3[b]

[a] Slope of plots in Fig 4 for logarithmic form of Eqs 7 and 8, log (dependent variable) = $-S/T + \log P = -\Delta H/2.303R(1/T) + \log P$.

[b] Calculated from the difference of the above two values

CALCULATIONS

The equations for the prediction of hydrolysis of saturated solutions of aspirin anhydride under any conditions of pH, acetate buffer concentration, and temperature are given in the pseudo-zero order Eqs 5 and 6 where

$$k = k_o + k_{OH^-}[OH^-] + k'_{Ac}[C_2H_3O_2^-] \qquad \text{(Eq. 9)}$$

$$k_o = 10^{4\ 082}10^{-2\ 030/T} \qquad \text{Eq 10)}$$

$$k_{OH^-} = 10^{14\ 68}10^{-3,430/T} \qquad \text{(Eq 11)}$$

$$k'_{Ac} = 10^{4\ 363}10^{-1\ 674/T} \qquad \text{(Eq 12)}$$

$$[AA]_s = 10^{3\ 845}10^{-2\ 445/T} \qquad \text{(Eq 13)}$$

where the k_i values are corrected in these equations to 0% dioxane as based on the comparison of 0% and 10% dioxane values at 25° and the temperature dependence of the k_i in 10% dioxane (8) Equations 10 to 13 are given in the nonlogarithmic form,

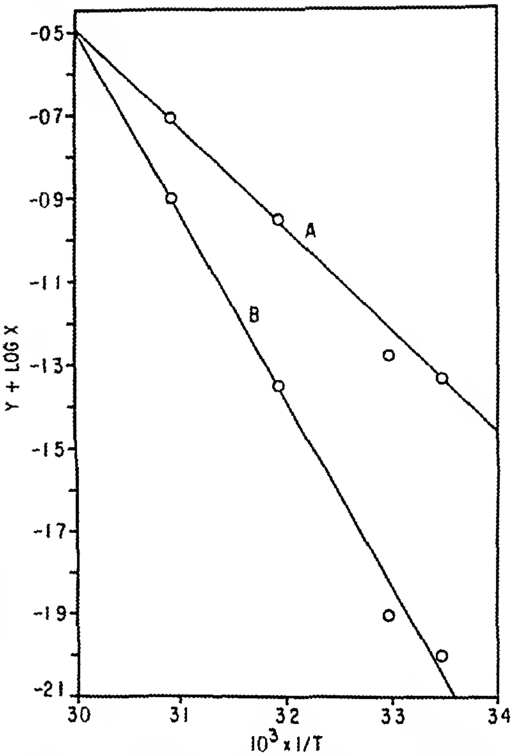


Fig 5.—Temperature dependence of aspirin anhydride solubility, $[AA]_s$ in M/1, and rate of anhydride hydrolysis, $-d[AA]/dt$ in M/1/sec , in 0 1 N HCl. Curve A, y = 3 and x = $[AA]_s$ Curve B, y = 5 and x = $-d[AA]/dt = -k[AA]_s$

$k_i = 10^{\log P}\ 10^{-S/T}$ of the Arrhenius equation, $\log k_i = \log P - S/T$, and T is the absolute temperature, where all k_i are in units of seconds

The rate of appearance of salicylic acid, [sal], is first order on aspirin, [asp], until the solution is saturated with aspirin (9, 10):

$$d[\text{sal}]/dt = k_{asp}[\text{asp}] \qquad \text{(Eq 14)}$$

where,

$$k_{asp} = k_1[H^+]/(1 + K'_a/[H^+]) + (k_4[H^+] + k_5C_{H_2O} + k_6[OH^-])/(1 + [H^+]/K'_a) \qquad \text{(Eq 15)}$$

$$k_1 = 10^{7.78}10^{-3,690/T} \qquad \text{(Eq 16)}$$

$$k_4 = 10^{9\ 05}10^{-3\ 590/T} \qquad \text{(Eq 17)}$$

$$k_5C_{H_2O} = 10^{7\ 45}10^{-3\ 840/T} \qquad \text{(Eq 18)}$$

$$k_6 = 10^{3\ 18}10^{-2\ 730/T} \qquad \text{(Eq 19)}$$

$$K'_a = 2\ 4 \times 10^{-4} \qquad \text{(Eq 20)}$$

The appearance of salicylic acid from aspirin anhydride is the result of a sequence of a zero-order and first-order reaction where:

$$[\text{asp}] + [\text{sal}] = 2kt \qquad \text{(Eq 21)}$$

Until all anhydride has disappeared, the rate of accumulation of aspirin is equal to the difference in its rate of formation from anhydride by Eqs 5 and 6 and its decomposition into salicylic acid by Eq 14 so that

$$d[\text{asp}]/dt = -d[AA]/dt - d[\text{sal}]/dt \qquad \text{(Eq 22)}$$

On integration, realizing that [asp] = 0 when $t = 0$,

$$[\text{asp}] = 2k(1 - e^{-k_{\text{asp}}t})/k_{\text{asp}} \quad \text{(Eq. 23)}$$

so that on the basis of Eqs. 21 and 23

$$[\text{sal}] = 2kt - 2k(1 - e^{-k_{\text{asp}}t})/k_{\text{asp}} \quad \text{(Eq. 24)}$$

Application of these equations at the physiological temperature of 37.5° gives the rate of anhydride hydrolysis as a function of pH from Eqs. 5, 9, 10, 11, and 13;

$$\begin{aligned} d[\text{AA}]/dt &= \{3.51 \times 10^{-3} + \\ &\quad 3.4 \times 10^{3}[OH^-]\}9.38 \times 10^{-5} \\ &= 32.9 \times 10^{-8} + \\ &\quad 0.32[OH^-] \text{ in } M/\text{L./sec.} \quad \text{(Eq. 25)} \end{aligned}$$

Below a pH of 6.0, the hydroxyl ion catalysis is insignificant so that the appearance of salicylic acid and aspirin on the basis of Eqs. 6, 21, and 25 would be

$$[\text{asp}] + [\text{sal}] = 2.37 \times 10^{-3}t \quad \text{(Eq. 26)}$$

where t is in hours.

The rate of salicylic acid appearance from the hydrolysis of aspirin at 37.5° may be calculated from Eqs 14–20 where:

$$\begin{aligned} k_{\text{asp}}(\text{sec}^{-1}) &= \\ &\underbrace{7.15 \times 10^{-6}[H^+]/(1 + 2.4 \times 10^{-4}/[H^+])}_{a} + \\ &(\underbrace{3.06 \times 10^{-3}[H^+]}_{b} + \underbrace{1.21 \times 10^{-5}}_{c} + \\ &\underbrace{0.24\ [OH^-]}_{d})/(1 + 4.17 \times 10^{3}[H^+]) \quad \text{(Eq. 27)} \end{aligned}$$

The items in Eq. 27 have no significant contribution under the following pH conditions: a above 3.0, b above 4.0, c below 1.0 and above 11.0, and d below 8.0. Obviously, item d is of no physiological consequence.

At a pH of 1.0, the probable maximum acidity of the stomach, the salicylic acid concentration from a saturated solution of aspirin anhydride in excess as a function of time can be derived from Eq. 24 where t is in hours.

$$\begin{aligned} [\text{sal}] &= 2.37 \times 10^{-3}t - \\ &\quad 0.0834(1 - e^{-0.0284t}) \quad \text{(Eq. 28)} \end{aligned}$$

Salicylic acid appearance from a saturated solution of aspirin in excess as a function of time at 37.5° may be derived from the knowledge of the k_{asp} values of Eq. 27 and the solubility of aspirin at 37.5° where $[\text{asp}]_s = 3.16 \times 10^{-2}$

$$[\text{sal}] = k_{\text{asp}}t \quad \text{(Eq. 29)}$$

where t is in hours and $k_{\text{asp}} = 8.98 \times 10^{-4}\ M/\text{L./hr.}$ at pH 1.0.

A comparison of salicylic acid production from saturated solutions of aspirin and aspirin anhydride at pH values of 0.5 and 1.0 over twenty hours is given in Fig. 6 as based on Eqs. 28 and 29. Similar relationships between salicylic acid concentrations from these sources will hold for all pH values.

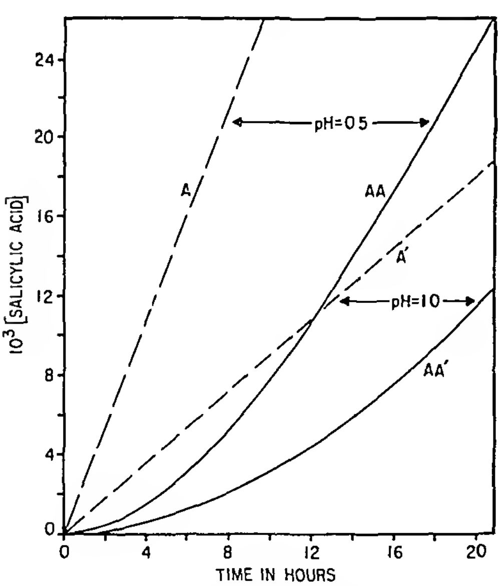


Fig. 6.—Comparison of salicylic acid production from saturated solutions of aspirin (curves A, A′) and aspirin anhydride (curves AA, AA′) at pH 0.5 (curves A, AA) and pH 1.0 (curves A′, AA′) at 37.5°.

DISCUSSION

Advantages of Anhydride Over Aspirin Relative to Gastric Irritation.—The prime disadvantages of aspirin therapy, those of digestive upset and gastric mucosal irritation, are well known. The literature (12) has provided strong evidence that aspirin is a gastric mucosal irritant.

As developed in the introduction, this irritation may be due to (*a*) salicylic acid production, (*b*) the natural acidity of aspirin, or (*c*) the adhesion of undissolved acidic aspirin to the gastric mucosa. A nonacidic "aspirin" would be ideal in that the rational causes of these disadvantageous phenomena would not be present. Aspirin anhydride fills the bill in that (*a*) its salicylic acid production under the pH conditions of the stomach (13) or the intestine is negligible compared to that from aspirin (see Fig. 6), (*b*) aspirin anhydride is nonacidic, and (*c*) aspirin anhydride has to dissolve to give aspirin, which when in solution may be almost instantaneously absorbed into the blood (11) so that the adhesion of irritating aspirin to the gastric mucosa cannot readily occur.

Advantages of Anhydride Over Aspirin Relative to Intestinal Absorption and Prolonged Action.—Feinblatt and Ferguson (14) have shown that within four minutes after the disintegration of a capsule in a fasted stomach, the capsule contents are in the process of leaving the stomach. Edwards (11) has measured the dissolution of aspirin, with agitation, of the 500-cc. covering liquid and has shown that, under these excellent solution-mixing conditions, only 0.6 of the aspirin in a 5-gr. tablet can be dissolved in three minutes at pH values of 1 to 2. This clearly indicates that with the less ideal agitation afforded by the empty or resting stomach, even less of the aspirin will be solubilized in the stomach, that a large fraction of the aspirin will find its way into the upper intestine prior to

solution, and even more than this fraction will enter the duodenum prior to absorption into the blood.

Edwards (11) has concluded that the absorption process of aspirin is extremely rapid and could be regarded as substantially complete within a few minutes of the aspirin dissolving; and that for aspirin taken in tablet form the rate-controlling process, for analgetic action resulting from blood concentration, is the dissolution within the stomach and intestine. The assumption was made that transfer rate was diffusion controlled rather than equilibrated with the blood on the blood side of the lipoid barrier. This may not be valid, since aspirin maxima in the blood are reached two hours after ingestion (15). Edwards considered the general case of all aspirin being able to diffuse which according to the Brodie, Hogben, Shore studies (16) is not so, only the undissociated aspirin may diffuse through the lipoid barrier.

It can be shown (Appendix, case C) that the general expression for the calculation of the concentration of an ionizable material in the blood, C_2, at time t is:

$$\log C_0 \Big/ \left\{ C_0 - C_2 \left[\left(\frac{1-\alpha_2}{1-\alpha_1} \right) + V/v \right] \right\} =$$
$$0.434\, DAt\{[1-\alpha_2]v + [1-\alpha_1]V\}/hvV \quad \text{(Eq. 30)}$$

where C_0 is the concentration in the gastrointestinal tract in a volume, v, where the material has an α_1 degree of dissociation, where the volume of the blood, V, is in instantaneous equilibrium and the degree of dissociation therein is α_2; where D is the diffusion constant, and h is the thickness, and A the effective area of the epithelial layer. On use of Edwards' parameters the Eq. 30 becomes for two 5-gr. tablets dispersed in the upper intestine or jejunum in a volume of v = 500 cc.

$$\log 1.3 \Big/ \left\{ 1.3 - C_2 \left[\left(\frac{1-\alpha_2}{1-\alpha_1} \right) + 6 \right] \right\} =$$
$$4.17 \times 10^{-3} t \;\; \{(1-\alpha_2)0.5 + (1-\alpha_1)3\} \quad \text{(Eq. 31)}$$

where t is in seconds.

Two probable conditions to consider are pH 6 or 7 for the intestine in equilibrium with material at pH 7 of the blood. The degrees of dissociation, α_1 and α_2, may be calculated at these pH values for aspirin of a $pK'_a = 3.8$ by

$$\alpha = \text{antilog}\,(\text{pH} - pK'_a)/[1 + \text{antilog}\,(\text{pH} - pK'_a)] \quad \text{(Eq. 32)}$$

so that Eq. 31 reduces to the form

$$\log [1.3/(1.3 - qc_2)] = rt \quad \text{(Eq. 33)}$$

where t is in minutes and if the intestinal contents are at pH 6.0, $1\text{-}\alpha_1 = 6.2 \times 10^{-3}$, $q = 6.1$, $r = 4.73 \times 10^{-3}$, and if the intestinal contents are at pH 7.0, $1\text{-}\alpha_1 = 6.2 \times 10^{-4}$, $q = 7$, and $r = 5.21 \times 10^{-4}$.

The aspirin content of the blood, VC_2, for pH 6 and 7 of the intestine, as a function of time is shown in Fig. 7 for one and two 5-gr. tablets in 500 cc. of intestinal volume.

The concentration of total salicylate (aspirin and aspirin anhydride) in the blood for a saturated solution of aspirin anhydride, C_s = 0.03211 Gm./L. at 37.5°, in an intestinal volume, v = 500 cc., is w/V where w may be given by (see Appendix, case B):

$$w = \frac{VC_sDA}{[Vhk + DA]} \left\{ kt + \frac{DA}{[Vhk + DA]} \left(1 - e^{-[(k+DA/Vh)t]}\right) \right\} \quad \text{(Eq. 34)}$$

and where $k = 3.85 \times 10^{-3}$ sec.$^{-1}$ is the first-order rate constant for the hydrolysis of aspirin anhydride.

On use of Edwards' parameters (11) Eq. 34 becomes

$$w(\text{Gm.}) = 0.0123t + 0.0296(1 - e^{-0.619t}) \quad \text{(Eq. 35)}$$

where t is in minutes. The contribution of aspirin anhydride to the total salicylate concentration in the blood will not exceed 1.78×10^{-5} Gm./cc. since it is so readily hydrolyzed in solution to aspirin. The rate of diffusion of aspirin anhydride through the intestinal wall is constant for this steady state condition reached within ten minutes and is (see Appendix, case C).

$$w\,(\text{Gm.}) = 0.0123t \quad \text{(Eq. 36)}$$

where t is in minutes.

The calculated values plotted in Fig. 7 clearly show that aspirin anhydride in the intestine provides aspirin to the blood at a much faster rate than aspirin at concentrations given by two or one 5-gr. tablets per 500 cc. of intestinal volume, at intestinal pH values of 6 or 7.[1] The total acetylsalicylate appearance from aspirin anhydride is independent of the intestinal pH, volume, or number of tablets in the intestine; its rate is a constant as long as sufficient anhydride is available to exceed the low solubility. The pH of the intestine ranges from 6 to 7 and thus it can be concluded that the acetylsalicylate appearance rate from aspirin is at least intermediate between curves B and D of Fig. 7.

Table III gives salicylate blood levels at several time intervals on the basis of different intestinal conditions. Metabolic elimination was assumed to be inoperative from both the gastrointestinal tract and the blood in the calculation of these values.

Figure 7 and Table III may not give the true

TABLE III.—ACETYLSALICYLATE CONTENT OF pH 7 BLOOD IN GM./L. AS A FUNCTION OF TIME FROM ASPIRIN AND ASPIRIN ANHYDRIDE IN THE INTESTINE

	Gr./500 cc. Intestinal Volume			
	Aspirin			Aspirin Anhydride
	10	5	10	Any[a]
	Intestinal pH			
Time, Min.	6	6	7	Any
5	0.0113	0.0057		0.030
10	0.0220	0.0110	0.0022	0.051
60	0.102	0.051	0.013	0.256
100	0.141	0.071	0.021	0.420
1,000	0.212[b]	0.106[b]	0.130	4.11
10,000			0.185[b]	

[a] Total transfer of 5 gr. of aspirin anhydride is accomplished within twenty-four minutes, of 10 gr. within fifty minutes. Hydrolysis to aspirin in intestine and metabolic elimination are ignored.

[b] Maximum blood concentration achievable due to the necessary equilibrium between intestinal contents and blood. Metabolic elimination is ignored.

[1] Dr. W. L. Miller, Jr., of these laboratories has confirmed this prediction of higher aspirin blood levels from aspirin anhydride oral administration over aspirin oral administration in humans and will publish his findings shortly.

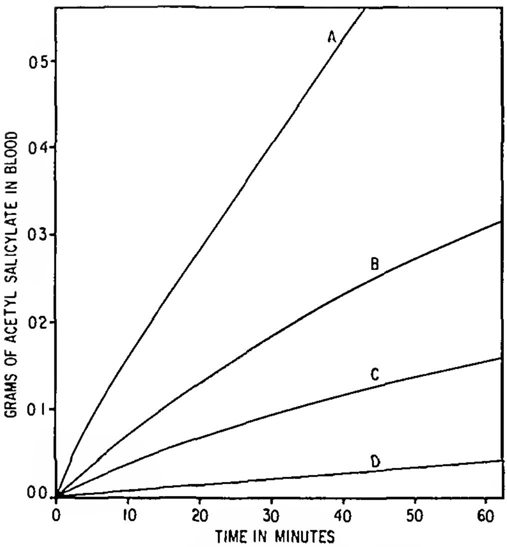


Fig. 7.—Appearance of acetylsalicylate in the blood from aspirin and its anhydride at several intestinal pH values and concentrations. Curve A is for aspirin anhydride at any pH and at any dosage in excess of saturation. Curve B is for two 5-gr. tablets of aspirin in 500 cc. of intestinal fluid at pH of 6 whereas curve C is for one such tablet. Curve D is for two 5-gr. tablets of aspirin in 500 cc. at an intestinal pH of 7.

values of blood concentration because of the probable invalidity of the assumption that the blood is in instantaneous equilibrium with the material in the lipoid barrier.. However, the relative values should be valid for comparison of total acetylsalicylate blood levels from aspirin and aspirin anhydride. However, the aspirin maxima in the blood from aspirin ingestion calculated on these bases are reached in about two hours after ingestion. This fact is consistent with experimental evidence, with the postulated importance of intestinal absorption, and the pH dependence of aspirin absorption in the intestine. It is inconsistent with the results of Edwards' premise (11) of pH independent absorption in the gastrointestinal tract, that aspirin absorption into the blood is virtually instantaneous.

SUMMARY AND CONCLUSIONS

1. Aspirin anhydride, as well as being a non-acidic drug, fulfills other rational criteria for a superior aspirin dosage form. From the physicochemical evidence: (*a*) Aspirin blood levels from aspirin anhydride will be higher than from aspirin ingestion due to the latter's relatively slow, pH-dependent absorption from the intestine. This should be most pronounced at low stomach retention times, e. g., with fasted individuals. (*b*) The salicylic acid content in the gastrointestinal fluids and in the blood will be higher at any time from aspirin ingestion than from the ingestion of aspirin anhydride. This is due to the routes of hydrolysis of these two substances.

2. The heterogeneous hydrolysis of aspirin anhydride has been studied at several temperatures and its aqueous solubilities have been determined. Complete equations have been presented for the hydrolysis of this material at the physiological temperatures and pH values.

3. Procedures have been developed to determine aspirin, aspirin anhydride, and salicylic acid spectrophotometrically.

4. The solubility equilibrium procedure presented will permit the estimation of aspirin in aspirin anhydride without the need of a primary standard. The procedure has been applied.

APPENDIX

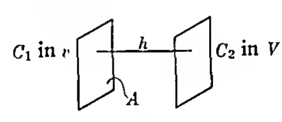
Derivation of Equations for Calculation of Diffusion of Amounts of Various Aspirin Derivatives from the Intestine into the Blood

Edwards (11) has assigned values to the necessary parameters to calculate the concentration of aspirin in the blood on diffusion through the intestinal wall. He concluded that the rate-controlling process for analgetic action in the blood is the dissolution of aspirin within the stomach and intestine. However, the basic premise of his calculations and conclusions was that all aspirin in the intestine diffuses into the blood stream. This is not believed to be the case; only undissociated aspirin may be presumed to diffuse through the lipoid barrier (16).

The General Case.—Fick's law of diffusion (17) states that the quantity dw diffusing across A cm.^{2} of area in time dt with a concentration gradient of $(C'_2 - C'_1)/h$ may be expressed mathematically by

$$dw/dt = -AD(C'_2 - C'_1)/h \quad \text{(Eq. 1)}$$

The following figure shows that w Gm. of material can be transported across h cm. of intestinal wall with a total area of A cm.^{2} and a diffusion constant of D cm.^{2}/sec. when the total concentration of the material is C_1 Gm./cc. in a volume, v, in the intestine, and where the concentration is C_2 Gm./cc. in a volume V of the blood.



The material balance in the intestine and blood can be expressed by

$$vC_0 = vC_1 + VC_2 \quad \text{(Eq. 2)}$$

where C_0 is the original concentration of the material in the intestine at time $t = 0$, prior to diffusion.

All calculations are to be based on Edwards' parameters, i. e., two 5-gr. tablets are dispersed in the upper intestine or jejunum in a volume of $v = 500$ cc., giving an apparent $C_0 = 1.3 \times 10^{-3}$ Gm./cc., where $D = 1.2 \times 10^{-5}$ cm.^{2}/sec., $A = 3 \times 10^3$ cm.^{2}, $V = 3 \times 10^3$ cc., and $h = 2.5 \times 10^{-3}$ cm., and homogeneous dispersion in the blood is assumed.

Case A. Diffusion Through the Intestinal Wall of Undissociated Ionizable Materials.—The amount

diffused through the intestinal wall at a time t is

$$w = C_2 V \qquad \text{(Eq. 3)}$$

but the actual concentration gradient is set up by undissociated molecules so that if α_1 is the degree of dissociation at the pH of the intestine and α_2 at the pH of the blood, then the effective concentration of diffusable materials is $(1 - \alpha_i)C_i$ so that the concentration gradient in this case is actually

$$(C'_2 - C'_1)/h = [(1 - \alpha_2)C_2 - (1 - \alpha_1)C_1]/h \qquad \text{(Eq. 4)}$$

Substitutions of eqs. 3 and 4 into 1 and using the value of C_2 as calculated from Eq. 2, and rearranging in integrable form, we have

$$\int_0^{C_2} \frac{dC_2}{[1 - \alpha_1]vC_0 - C_2[[1 - \alpha_2]v + [1 - \alpha_1]V]} = \int_0^t \frac{DA}{hvV}\, dt \qquad \text{(Eq. 5)}$$

which integrates to

$$\ln C_0 \Big/ \left\{C_0 - C_2\left[\left(\frac{1 - \alpha_2}{1 - \alpha_1}\right) + V/v\right]\right\} = DAt\{[1 - \alpha_2]v + [1 - \alpha_1]V\}/hvV \qquad \text{(Eq. 6)}$$

Substitution of the appropriate numerical values cited permits the simplification of Eq. 6 to

$$\log 1.3 \Big/ \left\{1.3 - C_2\left[\left(\frac{1 - \alpha_2}{1 - \alpha_1}\right) + 6\right]\right\} = 4.17 \times 10^{-3}t[(1 - \alpha_2)0.5 + (1 - \alpha_1)3] \qquad \text{(Eq. 7)}$$

Case B. Diffusion Through the Intestinal Wall of Material at Constant Soluble Concentration, C_s, in the Intestinal Contents, Which Is Transformed at a Finite Rate to Nondiffusible Material in the Blood.—If the concentration of diffusible material in the intestine is a constant, i. e., $C'_1 = C_s = 3.2 \times 10^{-5}$ Gm./cc. (solubility of AA at 37.5°) as when the amount available exceeds its solubility, and if this material is readily transformed in the blood at concentration C_2 to a nondiffusible charged material of concentration C_3 by first-order kinetics, then

$$dC_3/dt = kC_2 \qquad \text{(Eq. 8)}$$

where $k = 3.85 \times 10^{-3}$ sec.$^{-1}$ at 37.5° and the concentration gradient in this case is

$$(C'_2 - C'_1)/h = (C_2 - C_s)/h \qquad \text{(Eq. 9)}$$

The amount diffused through the intestinal wall at a time t is

$$w = [C_2 + C_3]V \qquad \text{(Eq. 10)}$$

so that on substitution of Eqs. 9 and 10 into 1

$$dw/dt = Vd[C_2 + C_3]/dt = -DA(C_2 - C_s)/h \qquad \text{(Eq. 11)}$$

When Eq. 8 is used, rearrangement results in the linear equation

$$dC_2/dt + [k + (DA)/(Vh)]C_2 = (DAC_s)/(Vh) \qquad \text{(Eq. 12)}$$

The integrating factor is $e^{\int [k + (DA)/(Vh)]dt} = e^{[k + (DA)/(Vh)]t}$ so that Eq. 12 may be integrated between the limits 0 and C_2, 0 and t, rearranged and simplified to

$$C_2 = \frac{DAC_s}{[Vhk + DA]}\{1 - e^{-[k + (DA)/(Vh)]t}\} \qquad \text{(Eq. 13)}$$

Substitution of the appropriate numerical values cited permits the simplification of Eq. 13 to

$$C_2 = \frac{1.2C_s}{(250k + 1.2)}\{1 - e^{-[k + 4.8 \times 10^{-3}]t}\} = 1.78 \times 10^{-5}\{1 - e^{-8.65 \times 10^{-3}t}\} \qquad \text{(Eq. 14)}$$

To calculate the total amount, w, of material that passed through the membrane, Eq. 13 or 14 may be substituted into Eq. 11 and integrated between the limits 0 and w, 0 and t,

$$w = \frac{VC_sDA}{[Vhk + DA]}\left\{kt + \frac{DA}{[Vhk + DA]}(1 - e^{-[k + DA/Vh]t})\right\} = \frac{VDA}{[Vhk + DA]}\{C_skt + C_2\} \qquad \text{(Eq. 15)}$$

Substitution of the appropriate numerical values cited permits the simplification of Eq. 15 to

$$w = \frac{108C_s}{7.5k + 3.6 \times 10^{-2}}\left\{kt + \frac{3.6 \times 10^{-2}}{7.5k + 3.6 \times 10^{-2}}(1 - e^{-[k + 4.8 \times 10^{-3}]t})\right\} = 20.5 \times 10^{-5}t + 2.96 \times 10^{-2}(1 - e^{-8.65 \times 10^{-3}t}) \qquad \text{(Eq. 16)}$$

Case C. The Steady State of Case B.—When $t > 600$ sec., the second factor in Eq. 14 approaches unity so that the steady state concentration of material not transformed to C_3 is

$$C_2 = 1.2C_s/(250k + 1.2) = 1.78 \times 10^{-5}\ \text{Gm./cc},\ t > 600 \qquad \text{(Eq. 17)}$$

Thus, the steady state diffusion through the intestinal wall from Eq. 11 becomes

$$dw/dt = 20.4 \times 10^{-5}\ \text{Gm./sec.} \qquad \text{(Eq. 18)}$$

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Prediction of Stability in Pharmaceutical Preparations VI*

Stability, Products, and Mechanism in the Pyrolytic Degradation of Aspirin Anhydride

By EDWARD R. GARRETT, EDWARD L. SCHUMANN, and MARVIN F. GROSTIC

The effects of crystallizing solvent and moisture on the pyrolytic degradation of aspirin anhydride were studied by infrared spectroscopy. The unexpected effects of "soft glass"-catalyzed surface decomposition were observed. Micronization, drying, and avoidance of contact with alkali increased the thermal stability. A mechanism of acyl transfer in the formed melt adequately explains the products identified by isolation and infrared studies.

THE APPLICATION OF prediction stability to pharmaceuticals that degrade by solvolytic processes is generally quite straightforward The degradation may usually be assigned to pseudo zero- or first-order kinetics and the classical laws of physicochemical kinetics are quantitatively applicable (1–5). When solid materials, as powders or in admixtures of powders, are studied for stability prediction, the manner and rates of degradation may be more complex Appropriate treatment of the data may not give as elegant a quantitative prediction as for materials in solution but can give good estimates and proper ranking of various alternate preparations

Properly designed, frequently assayed accelerated stability studies may permit the evaluation of assay methods and of artifacts giving erroneous assay.

There is physical chemical evidence that aspirin anhydride is a superior form for the oral administration of aspirin (6, 7) Since logical dosage forms are as tablets or powders, the prediction of stability in these forms is of interest This paper reports on these stability studies and the products and mechanism in the pyrolytic degradation of aspirin anhydride

EXPERIMENTAL

Initial Design for Study of Pyrolytic Stability of Aspirin Anhydride in Soft Glass.—Twenty-five milligrams of aspirin anhydride were weighed out in 15 × 45-mm glass vials (Kimble Opticlear) with press fit polyethylene caps Twenty of each lot were placed in large sealed ointment-type jars each of which was submerged beneath the oil of 70, 60, 50, and 40° constant temperature baths Ten vials of each lot were left at room temperature and in a refrigerator at 4°.

The material was assayed by dissolution in 5 cc. $CHCl_3$ and evaluation of the resultant infrared spectra in the 1,600–1,850 cm^{-1} region The spectral carbonyl region of possible hydrolytic or other end products of aspirin anhydride degradation, i e, acetic anhydride, aspirin (asp), and salicylic acid (sal) is given in Fig 1 Estimates of the nonpyrolyzed purity of aspirin anhydride (AA) have been made from a 3-component assay at 1,782 cm^{-1}, 1,700 cm^{-1}, and 1,668 cm^{-1} using supposedly pure components as standards

The first three lots studied in this manner are given in Table I

Pyrolytic Degradation of Aspirin Anhydride in Soft-Glass Vials.—A typical example of the change of carbonyl spectra with time on the pyrolytic degradation of aspirin anhydride is given in Fig 2, i e, lot I at 60° Three pertinent wavelengths for observation are obviously at 1,787 5 (where absorbance decreases on pyrolysis), 1,737 5, and 1,700 cm^{-1} (where absorbances increase on pyrolysis) An apparent isobestic point can be observed at 1,755 cm^{-1}

TABLE I —INITIAL PYROLYTIC STUDIES ON ASPIRIN ANHYDRIDE

Lot	Comments	Recrystallization Solvent	H_2O^a, %
I	Original I R assay 100 0%, negligible asp and sal	Isopropyl alcohol-acetone	1 21
II	Salvage material from many lots. I R assay 94 6% AA, 0.6% asp, negligible sal	Isopropyl alcohol acetone	No weight loss
III	Purity >99% by solubility analysis in ethylacetate-*n*-heptane, 1·10, 100.1% AA by I R	Diethyl ether	No weight loss

^{a} By weight loss at 0 01 mm Hg, twenty-five hours at room temperature

* Received May 21, 1959, from The Research Division, The Upjohn Co, Kalamazoo, Mich
The prior paper in this series is given in reference (1)
The authors are greatly indebted to Mrs Lillian G Snyder and Mr George E Bronson for excellent technical assistance and to Mr W A Struck and his associates in our Analytical Laboratory for the analytical data
Presented to the Scientific Section, A PH A, Cincinnati meeting, August 1959

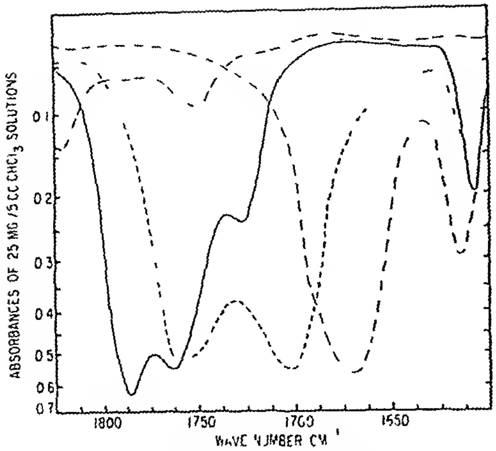


Fig 1—The carbonyl region infrared spectra of aspirin anhydride and hydrolytic products of amounts equivalent to 25 mg aspirin anhydride in 5 cc $CHCl_3$ aspirin anhydride ——, aspirin , salicylic acid - - -, acetic anhydride -

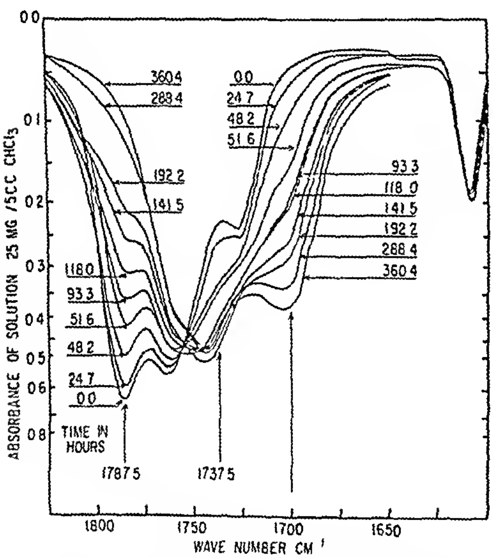


Fig 2—The carbonyl region infrared spectra of aspirin anhydride (lot I) degraded at 60° in "soft" glass vials The amount of aspirin anhydride was 25 mg , the spectra were run in 5 cc of $CHCl_3$

Comparison of Fig 2 with Fig 1 definitely shows that salicylic acid is not a product of the pyrolytic degradation since there is no appearance of a 1,668 cm $^{-1}$ salicylic acid band in Fig 2

Although the aspirin spectra of Fig 1 may account for part of the end product at three hundred and sixty hours, it obviously does not account for the whole since the absorbance at 1,700 cm $^{-1}$ is much less than the absorbance at 1,750 cm $^{-1}$ in Fig 2 These absorbances are almost equivalent for aspirin in Fig 1

The three hundred and sixty-hour spectra (Fig 2) subsequently changed little with time The nature of any change was a decrease in absorbances at all frequencies, indicating volatilization or loss of end product rather than any subsequent transformation

Typical plots of absorbance *vs* time at 1,787 5 cm $^{-1}$ are given in Fig 3 for the several temperatures of degradation The three lots, I, II, and III did not show any highly significant differences in 70 and 60° degradation and the plot of only one, lot I, is given in Fig 3 The curves did not fit zero-order or first-order type plots in their entirety The best characterization would be an initial linear loss of absorbance with a subsequent logarithmic or first order type loss

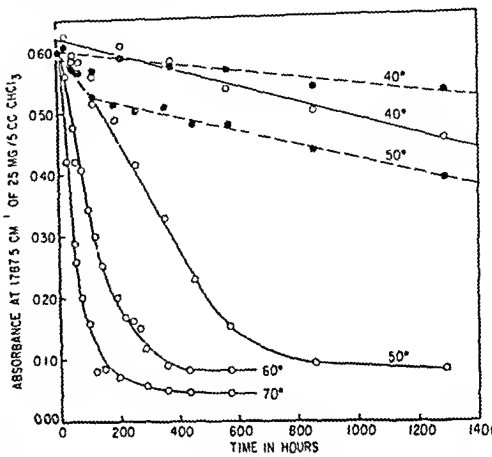


Fig 3—Pyrolytic degradation of typical aspirin anhydride lots in "soft" glass as measured by the absorbance of 25 mg in 5 cc $CHCl_3$ against time at 1,787 5 cm $^{-1}$, lot I ○, lot II ●

However, at 50 and 40° a distinct difference among lots in loss of absorbance with time can be observed Although lots II and III demonstrated similar degradation rates at these temperatures (only plots for lot II are shown in Fig 3), lot I degraded at a dramatically greater speed

Inspection of the rate of absorbance increases at 1,700 and 1,737 5 cm $^{-1}$ as given in Fig 4 showed similar phenomena All three lots similarly degraded at 70 and 60° and lots II and III degraded similarly at the other temperatures However, lot I degraded at a much faster rate than the other two This is most clearly shown in the 50° studies at these wave numbers

Visual observation of the degradation allowed the following conclusions The appearance of liquid occurred rather quickly and within twenty-four hours at 70° complete liquefaction had set in At the lower temperatures, change in carbonyl spectra could be qualitatively correlated with the degree of liquefaction, lot I being completely liquefied much before the others With decomposition, acetic acid or acetic anhydride could be smelled

In an attempt to manufacture a large amount of pyrolytic end product 10 Gm of lot I was sealed within a Carius tube and submerged in the 70° bath [1] Inspection of the curves (Fig 3) had indicated that complete transformation should have occurred within seven days The material was completely liquefied within two to three days However, an infrared spectrum of the resultant carbonyl region was not the same as that for three hundred and sixty hours at 60° in Fig 2 or four hundred and thirty-two

[1] This work was performed by Dr E C Olson of r laboratories

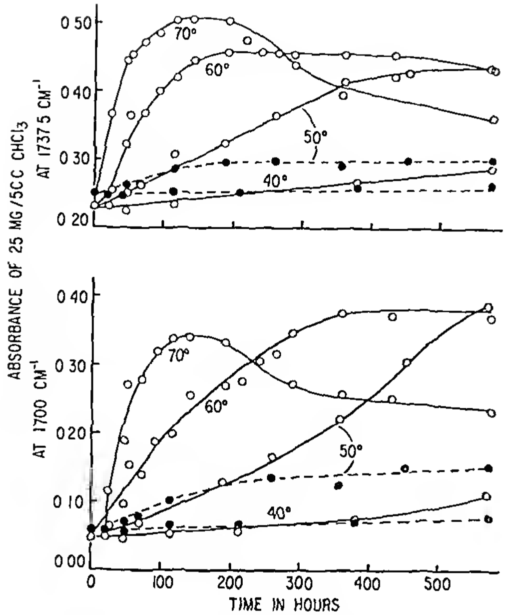


Fig. 4.—Pyrolytic degradation of typical aspirin anhydride lots in "soft" glass as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,700 and 1,737.5 cm.$^{-1}$; lot I ○; lot II ●.

hours at 70° in Fig. 5. The probably erroneous initial conclusion was that it was impure aspirin anhydride; the carbonyl region spectra were similar to that given for the anhydride of acetylsalicoylsalicylic acid in Fig. 6.

In addition, 10 Gm. of lot I were heated in a Pyrex tube at 70° for one week with a slow stream of air passing through the material. Volatile materials were trapped and identified as water and acetic acid.[1] The material did not liquefy. Infrared identified it as slightly decomposed aspirin anhydride.

Comparison of the Pyrolytic Stability of Aspirin Anhydride in "Soft" and "Hard" Glass Containers.—As previously, 25 mg. of aspirin anhydride was weighed out in the glass vials, i. e., "soft" glass. Similarly, the material was weighed out in Pyrex or so-called "hard" glass. These latter were sealed with corks wrapped in aluminum foil.

A dramatic difference was observed in the pyrolytic degradation of material in these two types of containers. This is graphically shown in Fig. 7 where the aspirin anhydride from the same lot pyrolyzed in "soft" glass showed large changes in carbonyl spectra at 1,787.5 cm.$^{-1}$ compared to those pyrolyzed in "hard" glass at both 70 and 50°.

Three lots (see Table II) of anydride showed no difference among themselves when pyrolyzed in "hard" glass at 50 and 70°. However, in "soft" glass, anhydride recrystallized from absolute ethanol showed a definitely worse stability (lot V) over material finally recrystallized from Skelly B-ether (lot IV) and Skelly B-ethyl acetate (lot VI). These facts indicate that ethanol of solvation is difficult to remove from the anhydride and may not be the recrystallization solvent of choice even though the nicest appearing and largest crystals resulted from this solvent.

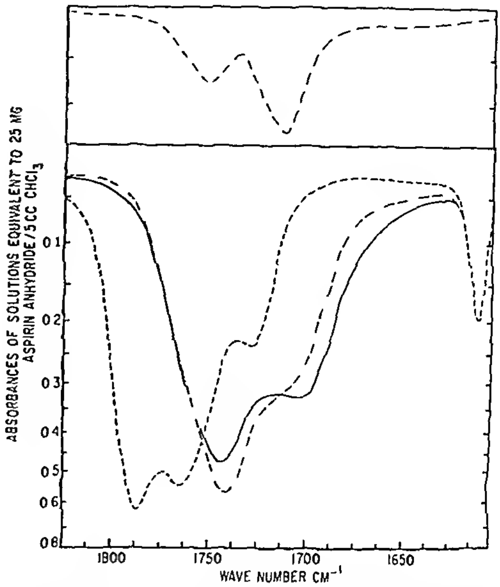


Fig. 5.—The carbonyl region infrared spectra of aspirin anhydride and possible pyrolytic end products of amounts equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride - - - -; end product of 70° pyrolysis at four hundred and thirty-two hours ——; acetylsalicoylsalicylic acid · · · · · · · ·; acetic acid - · - · -.

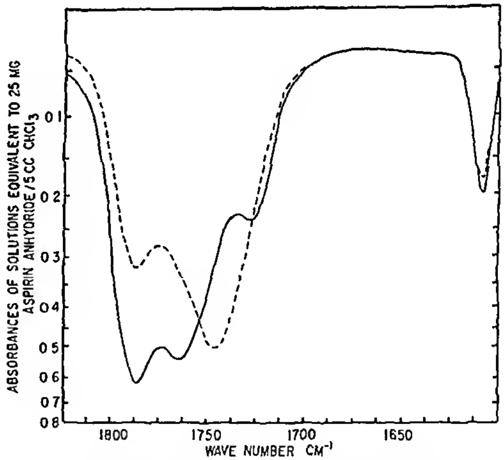


Fig. 6.—The carbonyl region infrared spectra of aspirin anhydride and a possible pyrolytic intermediate, equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride ——, 2,2'-bis(2-acetoxybenzoyloxy)benzoic anhydride - - - -.

To check the effect of the plastic cap, finely ground plastic caps were placed in contact with anhydride in the "hard" glass containers. No difference in degradation rate from the material alone in "hard" glass could be observed.

Comparison of Pyrolytic Stability of Micronized, Nonmicronized, and Impure Aspirin Anhydride.—The pyrolytic stability of aspirin anhydride was studied in screw-cap polyethylene containers. The material was subjected to varying temperatures and infrared-spectrophotometrically analyzed as previ-

ously described. Properties of the anhydride lots studied are tabulated in Table III.

No great difference in pyrolytic degradation among lots VII, IX, and X was observed. The plots of lot VII, absorbance at 1,787.5 cm.$^{-1}$ *vs.* time for 70, 60, and 50° in Fig. 8 is characteristic of all three of these lots. From this it can be concluded that the two different solvent mixtures of crystallization, ethyl aectate-Skelly B and acetone-isopropyl alcohol, are indistinguishable in their effects on the pyrolytic degradation of thoroughly dried aspirin anhydride. It is also inidcated that an admixture, containing up to 3% aspirin, has no enhanced pyrolytic degradation. However, it must be realized that this is not the same case as aspirin impurity molecularly dispersed throughout aspirin anhydride.

A synthetic admixture of anhydride with salicylic acid (i. e., 4.8%) does have significantly greater pyrolytic degradation rates than aspirin anhydride itself. The increased rates of degradation of lot XI over lot VII are clearly demonstrated in Fig. 8 at all three temperatures.

Micronized aspirin anhydride with subsequent thorough drying demonstrated highly significant increases in pyrolytic stability. Lot VIII, which is lot VII micronized and redried, shows an astonishing increase in stability over lot VII at all temperatures when the pertinent I. R. absorbances are plotted against time, as in Fig. 8.

Preparation of the Possible Products of Pyrolytic Degradation.—Six compounds were prepared for I. R. spectrophotometric comparison with thermally decomposed aspirin anhydride: *Acetylsalicoylsalicylic Acid, $C_{16}H_{12}O_6$, mol. wt. 300.3.*—This substance was prepared by the method of A. Einhorn

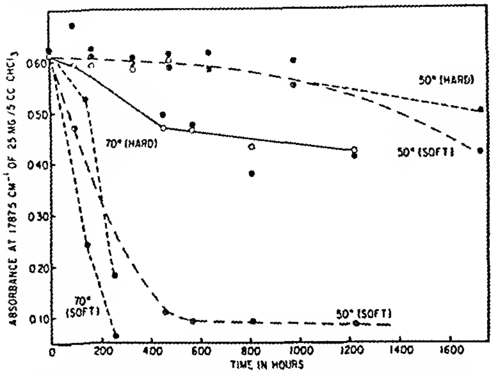


Fig. 7.—Comparative effects of Pyrex and "soft" glass containers on the pyrolytic degradation of aspirin anhydride as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,787.5 cm.$^{-1}$. The solid lines are for "soft" glass containers and the dashed are for Pyrex: lot IV ○; lot V ◑; lot VI ●.

TABLE II.—CHARACTERIZATION OF LOTS USED IN COMPARISON OF "SOFT" AND "HARD" GLASS CONTAINERS ON PYROLYTIC STABILITY OF ASPIRIN ANHYDRIDE

Lot	M. p. Uncorr., °C.	I. R. Assay AA, %	I. R. Assay Aspirin, %	Recrystallization Solvents
IV	84–85	101.0	0.1	3× from ethyl acetate-Skelly B 2:3; 1× from absolute ether-Skelly B 2:1
V	83–85	100.8	0.2	2× from ethyl acetate-Skelly B; 2:3; 1× from absolute ethanol
VI	84–86	100.3	0.0	2× from ethylacetate-Sk lly B; 2:3; 1× from acetone-Skelly B; 1:1; 1× from ethylacetate-Skelly B; 1:1

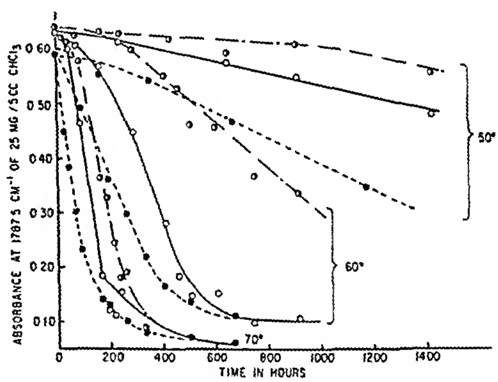
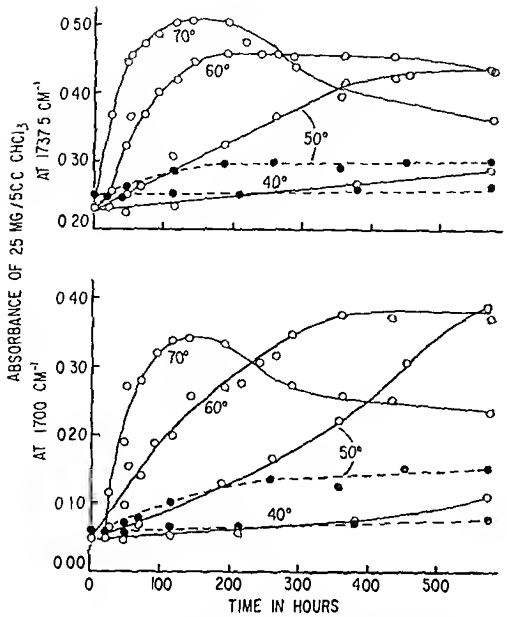


Fig. 8.—Comparison of the pyrolytic degradations of crystalline, micronized, and salicylic acid-contaminated aspirin anhydride in polyethylene bottles. The absorbance of 25 mg. in 5 cc. $CHCl_3$ was measured against time at 1,787.5 cm.$^{-1}$; lot VII, crystalline aspirin anhydride ○; lot VIII, micronized ◑; lot XI, aspirin anhydride plus 4.8% salicylic acid ●.

TABLE III.—CHARACTERIZATION OF LOTS USED IN COMPARISON OF MICRONIZATION, NONMICRONIZATION, AND ASPIRIN AND SALICYLIC ACID IN ADMIXTURES ON THE PYROLYTIC STABILITY OF ASPIRIN ANHYDRIDE

Lot	Recrystallization	Remarks	Assay by I. R.
VII and VIII	2× from ethyl acetate-Skelly B, 2:3	Dried overnight at room temperature at 2 mm. of Hg, then 24 hr. at 2 mm.; 0 02% wt. loss	99% AA 0% aspirin 0.1% salicylic acid
IX	Same as lot VII with a subsequent recrystallization 2× from acetone-isopropyl alcohol	Dried at 12 mm. for 24 hr., wt. loss 0.08%	101% AA 0.2% aspirin 0.1% salicylic acid
X	Synthetic batches derived from lot VII + salicylic acid + aspirin		96.6% AA 2.6% aspirin 0.3% salicylic acid
XI			95.1% AA 0.7% aspirin 4.8% salicylic acid



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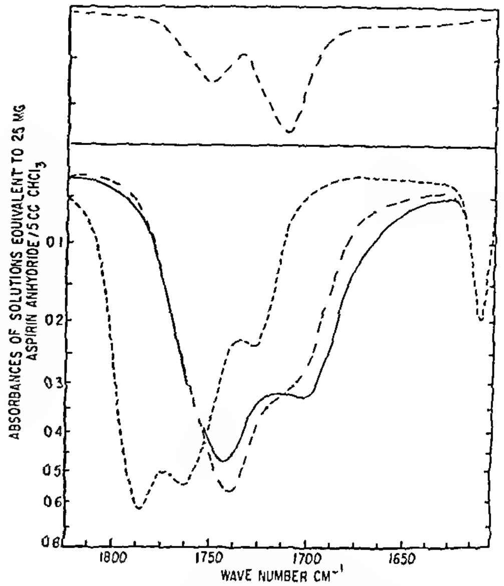

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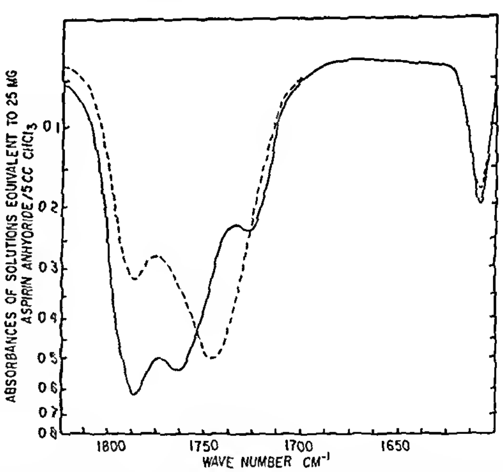

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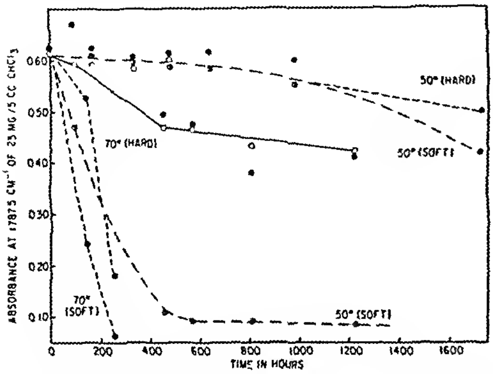

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that the two different solvent mixtures of crystallization, ethyl acetate-Skelly B and acetone-isopropyl alcohol, are indistinguishable in their effects on the pyrolytic degradation of thoroughly dried aspirin anhydride. It is also inidcated that an admixture, containing up to 3% aspirin, has no enhanced pyrolytic degradation. However, it must be realized that this is not the same case as aspirin impurity molecularly dispersed throughout aspirin anhydride.

A synthetic admixture of anhydride with salicylic acid (i. e., 4.8%) does have significantly greater pyrolytic degradation rates than aspirin anhydride itself. The increased rates of degradation of lot XI over lot VII are clearly demonstrated in Fig. 8 at all three temperatures.

Micronized aspirin anhydride with subsequent thorough drying demonstrated highly significant increases in pyrolytic stability. Lot VIII, which is lot VII micronized and redried, shows an astonishing increase in stability over lot VII at all temperatures when the pertinent I. R. absorbances are plotted against time, as in Fig. 8.

Preparation of the Possible Products of Pyrolytic Degradation.—Six compounds were prepared for I. R. spectrophotometric comparison with thermally decomposed aspirin anhydride: *Acetylsalicoylsalicylic Acid, $C_{16}H_{12}O_6$, mol. wt. 300.3.*—This substance was prepared by the method of A. Einhorn

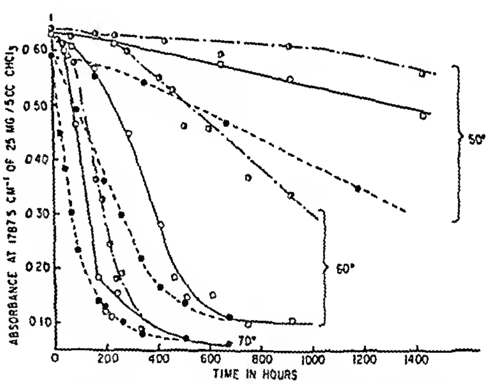

Fig. 8.—Comparison of the pyrolytic degradations of crystalline, micronized, and salicylic acid-contaminated aspirin anhydride in polyethylene bottles. The absorbance of 25 mg. in 5 cc. $CHCl_3$ was measured against time at 1,787.5 cm.$^{-1}$; lot VII, crystalline aspirin anhydride ○; lot VIII, micronized ◑; lot XI, aspirin anhydride plus 4.8% salicylic acid ●.

TABLE III.—CHARACTERIZATION OF LOTS USED IN COMPARISON OF MICRONIZATION, NONMICRONIZATION, AND ASPIRIN AND SALICYLIC ACID IN ADMIXTURES ON THE PYROLYTIC STABILITY OF ASPIRIN ANHYDRIDE

Lot	Recrystallization	Remarks	Assay by I. R.
VII and VIII	2× from ethyl acetate-Skelly B, 2:3	Dried overnight at room temperature at 2 mm. of Hg, then 24 hr. at 2 mm.; 0.02% wt. loss	99% AA 0% aspirin 0.1% salicylic acid
IX	Same as lot VII with a subsequent recrystallization 2× from acetone-isopropyl alcohol	Dried at 12 mm. for 24 hr., wt. loss 0.08%	101% AA 0.2% aspirin 0.1% salicylic acid
X	Synthetic batches derived from lot VII + salicylic acid + aspirin		96.6% AA 2.6% aspirin 0.3% salicylic acid
XI			95.1% AA 0.7% aspirin 4.8% salicylic acid

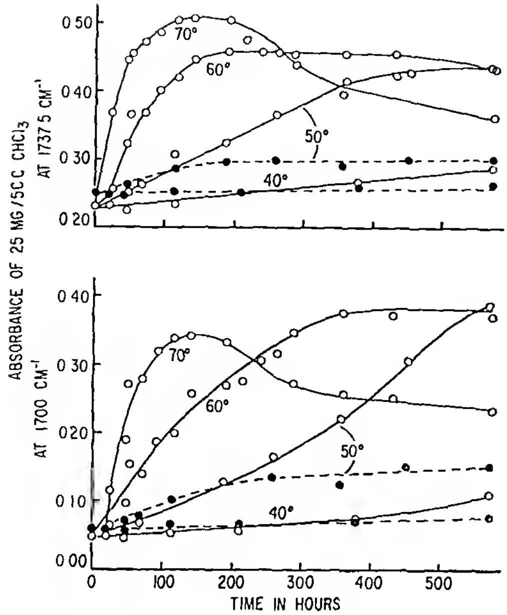

Fig. 4.—Pyrolytic degradation of typical aspirin anhydride lots in "soft" glass as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,700 and 1,737.5 cm.$^{-1}$; lot I ○; lot II ●.

hours at 70° in Fig. 5. The probably erroneous initial conclusion was that it was impure aspirin anhydride; the carbonyl region spectra were similar to that given for the anhydride of acetylsalicoylsalicylic acid in Fig. 6.

In addition, 10 Gm. of lot I were heated in a Pyrex tube at 70° for one week with a slow stream of air passing through the material. Volatile materials were trapped and identified as water and acetic acid.[1] The material did not liquefy. Infrared identified it as slightly decomposed aspirin anhydride.

Comparison of the Pyrolytic Stability of Aspirin Anhydride in "Soft" and "Hard" Glass Containers.—As previously, 25 mg. of aspirin anhydride was weighed out in the glass vials, i. e., "soft" glass. Similarly, the material was weighed out in Pyrex or so-called "hard" glass. These latter were sealed with corks wrapped in aluminum foil.

A dramatic difference was observed in the pyrolytic degradation of material in these two types of containers. This is graphically shown in Fig. 7 where the aspirin anhydride from the same lot pyrolyzed in "soft" glass showed large changes in carbonyl spectra at 1,787.5 cm.$^{-1}$ compared to those pyrolyzed in "hard" glass at both 70 and 50°.

Three lots (see Table II) of anydride showed no difference among themselves when pyrolyzed in "hard" glass at 50 and 70°. However, in "soft" glass, anhydride recrystallized from absolute ethanol showed a definitely worse stability (lot V) over material finally recrystallized from Skelly B-ether (lot IV) and Skelly B-ethyl acetate (lot VI). These facts indicate that ethanol of solvation is difficult to remove from the anhydride and may not be the recrystallization solvent of choice even though the nicest appearing and largest crystals resulted from this solvent.

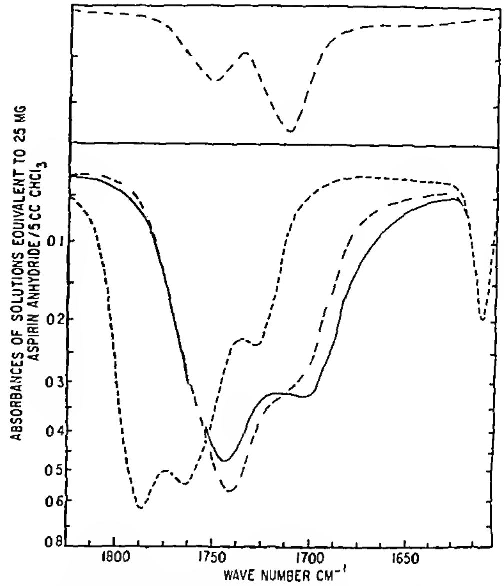

Fig. 5.—The carbonyl region infrared spectra of aspirin anhydride and possible pyrolytic end products of amounts equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride - - - -; end product of 70° pyrolysis at four hundred and thirty-two hours ——; acetylsalicoylsalicylic acid ········; acetic acid -·-·-.

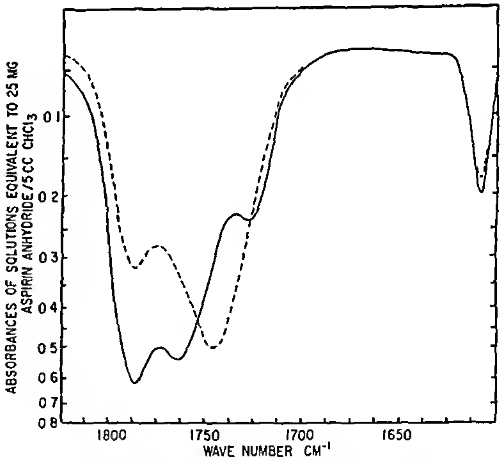

Fig. 6.—The carbonyl region infrared spectra of aspirin anhydride and a possible pyrolytic intermediate, equivalent to 25 mg. aspirin anhydride in 5 cc. $CHCl_3$: aspirin anhydride ——, 2,2′-bis(2-acetoxybenzoyloxy)benzoic anhydride - - - -.

To check the effect of the plastic cap, finely ground plastic caps were placed in contact with anhydride in the "hard" glass containers. No difference in degradation rate from the material alone in "hard" glass could be observed.

Comparison of Pyrolytic Stability of Micronized, Nonmicronized, and Impure Aspirin Anhydride.—The pyrolytic stability of aspirin anhydride was studied in screw-cap polyethylene containers. The material was subjected to varying temperatures and infrared-spectrophotometrically analyzed as previ-

ously described. Properties of the anhydride lots studied are tabulated in Table III.

No great difference in pyrolytic degradation among lots VII, IX, and X was observed. The plots of lot VII, absorbance at 1,787.5 cm.$^{-1}$ *vs.* time for 70, 60, and 50° in Fig. 8 is characteristic of all three of these lots. From this it can be concluded

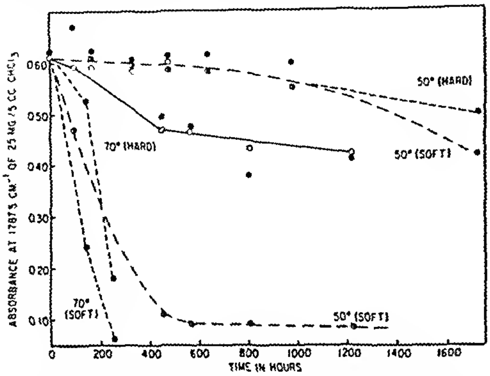

Fig. 7.—Comparative effects of Pyrex and "soft" glass containers on the pyrolytic degradation of aspirin anhydride as measured by the absorbance of 25 mg. in 5 cc. $CHCl_3$ against time at 1,787.5 cm.$^{-1}$. The solid lines are for "soft" glass containers and the dashed are for Pyrex: lot IV ○; lot V ◑; lot VI ●.

TABLE II.—CHARACTERIZATION OF LOTS USED IN COMPARISON OF "SOFT" AND "HARD" GLASS CONTAINERS ON PYROLYTIC STABILITY OF ASPIRIN ANHYDRIDE

Lot	M. p. Uncorr., °C.	I. R. Assay AA, %	I. R. Assay Aspirin, %	Recrystallization Solvents
IV	84–85	101.0	0.1	3× from ethyl acetate-Skelly B 2:3; 1× from absolute ether-Skelly B 2:1
V	83–85	100.8	0.2	2× from ethyl acetate-Skelly B; 2:3; 1× from absolute ethanol
VI	84–86	100.3	0.0	2× from ethylacetate-Sk lly B; 2:3; 1× from acetone-Skelly B; 1:1; 1× from ethylacetate-Skelly B; 1:1

that the two different solvent mixtures of crystallization, ethyl acetate-Skelly B and acetone-isopropyl alcohol, are indistinguishable in their effects on the pyrolytic degradation of thoroughly dried aspirin anhydride. It is also inidcated that an admixture, containing up to 3% aspirin, has no enhanced pyrolytic degradation. However, it must be realized that this is not the same case as aspirin impurity molecularly dispersed throughout aspirin anhydride.

A synthetic admixture of anhydride with salicylic acid (i. e., 4.8%) does have significantly greater pyrolytic degradation rates than aspirin anhydride itself. The increased rates of degradation of lot XI over lot VII are clearly demonstrated in Fig. 8 at all three temperatures.

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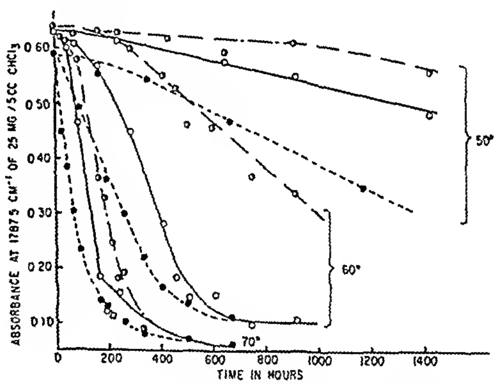

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IX	Same as lot VII with a subsequent recrystallization 2× from acetone-isopropyl alcohol	Dried at 12 mm. for 24 hr., wt. loss 0.08%	101% AA 0.2% aspirin 0.1% salicylic acid
X	Synthetic batches derived from lot VII + salicylic acid + aspirin		96.6% AA 2.6% aspirin 0.3% salicylic acid
XI			95.1% AA 0.7% aspirin 4.8% salicylic acid

(8) who reported a melting point of 161–162° Material obtained in this study melted at 163–165° (uncorr) after recrystallization from benzene and 95% ethanol

2,2' Bis(2 acetoxybenzoyloxy) benzoic Anhydride, $C_{32}H_{22}O_{11}$, *mol wt 582 4*—A mixture of 9 0 Gm (0 03 mole) of acetylsalicoylsalicylic acid in 15 ml of methylene chloride and 2 4 Gm (0 03 mole) of pyridine was stirred and cooled in an ice bath during the addition of a solution of 1 8 Gm (0 015 mole) of $SOCl_2$ in 10 ml of methylene chloride over a five minute period The mixture was maintained at 5–8° and stirred fifteen minutes longer, then poured over a mixture of 100 ml of crushed ice and 15 ml of 10% HCl and mixed thoroughly

The methylene chloride layer was separated, dried over $MgSO_4$, and evaporated The residue, which crystallized on standing, was recrystallized twice from ethyl acetate Skelly B (1 1) to give 5 7 Gm (65%) of fine, white needles, melting point 142–144° (uncorr)

Anal—Calcd for $C_{32}H_{22}O_{11}$, C, 65 98, H, 3 81 Found C, 65 95, H, 3 83

Mixed Anhydride of Aspirin and Acetic Acid, $C_{11}H_{10}O_5$, *mol wt 222 2*—This compound and the mixed anhydride of acetylsalicoylsalicylic and acetic acids are very labile, decomposing at room temperature to give the expected symmetrical anhydrides No satisfactory carbon hydrogen analyses were obtained due to decomposition and loss of varying amounts of acetic anhydride

A solution of 18 0 Gm (0 1 mole) of aspirin in 600 ml of anhydrous ether was treated, via a gas inlet tube, with excess ketene The ketene was produced in a Hurd generator (9)

Solvent was removed at room temperature by a dry nitrogen stream and the residual colorless oil was stored at 0° until used for I R analysis

Pyrolysis of the mixed anhydride was effected by heating the material on the steam bath under reduced pressure (*ca* 40 mm for 90 minutes) Trituration of the residual oil with 1 1 ethyl acetate Skelly B and seeding gave a 46% yield of aspirin anhydride

Mixed Anhydride of Acetylsalicoylsalicylic and Acetic Acids, $C_{18}H_{14}O_7$, *mol wt 342 3*—A solution of 6 0 Gm of acetylsalicoylsalicylic acid in 200 ml of anhydrous acetone was treated with ketene as described above and worked up in the same way The residual oil was stored at 0° until used

Material which was allowed to stand at room temperature decomposed to give 2,2' bis(2 acetoxybenzoyloxy) benzoic anhydride

Disalicylide and Trisalicylide—These products were prepared by pyrolysis of aspirin at 300–350° at 10–15 mm , using the procedure of Baker, Ollis, and Zealley (10)

Infrared analysis showed no trace of these compounds in any of the decomposed aspirin anhydride samples examined

Isolation of Acetylsalicoylsalicylic Acid from Decomposed Aspirin Anhydride—Acetylsalicoylsalicylic acid was isolated from decomposed moist aspirin anhydride, lot I, pyrolyzed at 70° for three weeks in the presence of soft glass, in the following manner

An ether solution of decomposed aspirin anhydride was extracted with 5% aqueous $NaHCO_3$ and the bicarbonate solution was acidified with acetic acid and extracted with ether The ether solution was dried over $MgSO_4$ and then evaporated to give crude acetylsalicoylsalicylic acid which was almost identical to authentic material

2,2'-Bis(2 acetoxybenzoyloxy) benzoic anhydride could not be isolated from the complex of aspirin anhydride decomposition products

DISCUSSION

Three aspects of these studies on aspirin anhydride are to be considered The first is the effect of moisture, solvent, alkali, purity, and micronization on the stability of aspirin anhydride and the conditions for maximum stability The second is a proposed mechanism of acyl exchange for the pyrolytic degradation of aspirin anhydride and its consistency with the observed changes in infrared spectra of the carbonyl region as compared to the infrared spectra of compounds that could result from acyl interchange The third is the further evidence from isolation of degradation products and comparison of complete infrared spectra

Stabilization of Aspirin Anhydride Against Pyrolytic Degradation—It is apparent that water contamination increases the thermal instability of aspirin anhydride even in amounts as low as 1% This is aptly demonstrated by the plots of Fig 3 for lots I and II

Material in contact with "soft," or high alkali content, glass has the greater pyrolytic instability This is demonstrated by the curves of Fig 7 Comparison of the curves of Fig 8 for polyethylene with those of the "soft" glass containers of Fig 6 also substantiates this point

Contamination with salicylic acid adversely affects the pyrolytic stability as can be seen in Fig 8

It can be concluded from the above facts and other observations given in "Experimental" that the pyrolytic degradation of aspirin is initiated on the surface of the crystals, that it is catalyzed by the higher alkali content of contacted "soft" glass, that the surface formed products lower the melting point, originally *ca* 84°, to form a melt The pyrolytic degradation proceeds at a faster rate within the melt

When aspirin anhydride contains moisture (as in lot I) or is contaminated by solvent (as presumed in lot V), liquefaction and thus pyrolytic degradation is accelerated Under these conditions and especially when alkali is present for catalysis, as in "soft" glass, no induction period may be readily apparent (Fig 3) However, when the anhydride is thoroughly dried, of high purity, and not in surface contact with alkali, its pyrolytic stability is greatly enhanced, as with lots VII and VIII in Fig 8, the induction period becomes obvious Any material, such as salicylic acid, which significantly lowers the melting point and abets liquefaction will favor instability

Removal of the initial products of pyrolytic degradation of aspirin anhydride, for example, acetic anhydride or acid, or unbound or released solvent as by a stream of heated air, inhibits liquefaction and stabilizes

The increased stability of micronized anhydride over unmicronized (lot VIII over VII in Fig 8) or of small crystals from ethyl acetate Skelly B over the large crystals from ethanol crystallization (lot VI over

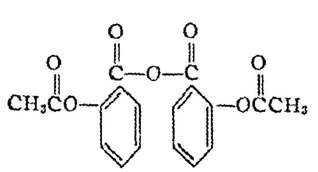
lot V in Fig. 7) are unexpected phenomena. This is especially so in the former case where lot VII showed negligible weight loss on drying (0.02%). A possible explanation is that large crystals are solvates or contain trapped solvent, that smaller or fractured crystals are more liable to lose this solvent of crystallization when subject to higher temperatures. Thus the melting points would not be lowered as much so that it is more difficult to produce the "melt" wherein pyrolytic degradation is accelerated.

Thus, to gain maximum stability to pyrolytic degradation, the aspirin anhydride should not be contaminated with salicylic acid, should be thoroughly dried, and micronization is highly desirable. Since contact with alkali or Bronsted-type bases may catalyze the decomposition, the absence of contacting alkaline surfaces should promote stabilization.

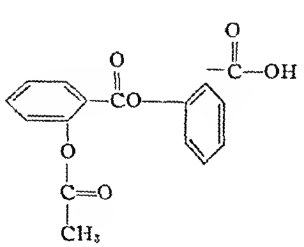
Removal of the products of pyrolysis, as by hot air stream, which could promote liquefaction and degradation will stabilize.

The studies on lot VII (Fig. 8), a dry pure material, show that for a 10° temperature decrease from 70 to 60°, the estimated linear degradation rate after the induction period decreased to $^1/_3$ its prior value. For the 10° decrease from 60 to 50° the similarly estimated linear degradation rate is decreased to $^1/_{10}$ its prior value. This indicates that the apparent overall heat of activation for the processes of pyrolytic degradation is of high magnitude. If a decrease to $^1/_{10}$ the value is postulated for the next 10° decrease in temperature, infrared assay should not be able to demonstrate any significant pyrolytic degradation for more than a year at 40°. Pure, dry aspirin anhydride should be indefinitely stable at 30° by this criterion. Consideration of the additional phenomenon of the lengthening induction period with lowered temperature makes this estimate a conservative one.

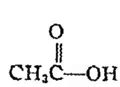
Proposed Mechanism for the Pyrolytic Degradation of Aspirin Anhydride.—Aspirin anhydride (I) with high moisture content (lot I) produced acetylsalicoylsalicylic acid (VII) and acetic acid (VIII) in large yield as the final products of pyrolytic degradation.



I



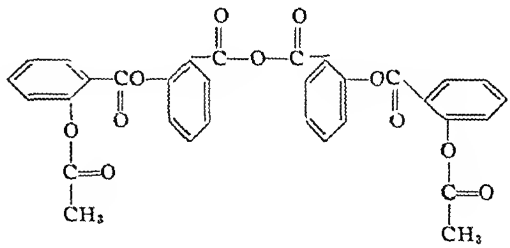
VII



VIII

The infrared spectra of the carbonyl region for aspirin anhydride pyrolytically degraded were highly consistent with the carbonyl region spectra of VII plus a partial contribution from the carbonyl spectra of acetic acid which was also present. This is clearly shown in Fig. 5.

When large amounts of dry aspirin anhydride were heated at 70° for a period of a week, a melt resulted. The carbonyl region spectra were not consistent with VII but with the anhydride of acetylsalicoylsalicylic acid (V) i. e., 2,2'-bis(2-acetoxybenzoyloxy)-benzoic anhydride.



V

Initial opinion as based on the I. R. was that the melt was slightly impure aspirin anhydride, I, since the 1,787.5 cm.$^{-1}$ band was still prominent although reduced in intensity and no band had appeared at 1,700 cm.$^{-1}$ which had been characteristic of the pyrolytic end products, i. e., VII (see Fig. 2). This is, of course, inconsistent with the fact of liquefac-

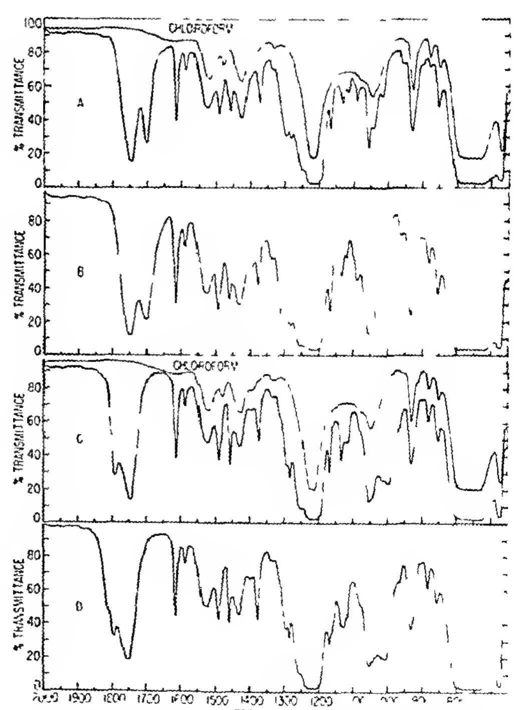


Fig. 9.—Partial infrared spectra in chloroform solution, concentration 0.5%. A.—acetylsalicoylsalicylic acid. B.—aspirin anhydride, lot I, completely degraded at 70°. C.—2,2'-Bis(2-acetoxybenzoyloxy)-benzoic anhydride. D.—anhydrous aspirin anhydride degraded at 70° for forty-eight hours in the presence of powdered soft glass. The dashed line is the absorption of the mixed anhydride of aspirin and acetic acid.

represents the pH of a saturated acetylsalicylic acid solution

Garrett (4) extended Edwards' study to several acyl esters of salicylic acid in an attempt to correlate the interdependence of solubility and rate of degradation of saturated solutions If the degradation of homogenous solution is first order, the saturated solution should be zero order

The hydrolysis of acetylsalicylic acid depends upon the amount of acetylsalicylic acid in solution, hence suspensions of acetylsalicylic acid show a low degree of hydrolysis relative to the total amount of drug in suspension K C James (5) found that a 6 per cent unbuffered aqueous acetylsalicylic acid suspension, after storage for forty seven days at room temperature contained 89 per cent of the drug

Various techniques have been recommended for preparing a more stable acetylsalicylic acid solution Clark (6) developed a formula containing potassium citrate that will give a "minimum of hydrolysis" Organic solvents such as ethanol, propylene glycol, and glycerin solubilize the acid, but the disadvantage to their use lies in the fact that most patients will not find any of these solvents palatable Schwarz, *et al* (7), have developed an acetylsalicylic acid elixir which, according to the authors, holds promise as a pediatric preparation The formula recommended uses as solvent a blend of ethanol and polyethylene glycol 400 At the end of twenty four days, 17 7 per cent of the acetylsalicylic acid hydrolyzed in this solvent

A recent formulation (8) of an acetylsalicylic acid suspension has been found " to be stable for one month without excessive hydrolysis" According to the authors, crystalline sorbitol, when used in combination with other ingredients exerted a pronounced stabilizing effect on the suspension Sorbitol has been used to stabilize vitamin C and B_{12} preparations (9), the action being attributed to two possible causes, the reduced availability of water in the sorbitol solution or complex formation between sorbitol and the drug

The objectives of this investigation were to study the kinetics of acetylsalicylic acid suspensions and the effects of various additives on the stability of the suspension

Higuchi, *et al* (10, 11), have reported on the interaction of acetylsalicylic acid and salicylic acid with several of the additives used in this study

EXPERIMENTAL

Reagents —Recrystallized acetylsalicylic acid, salicylic acid, calcium gluconate, reagent grade phosphoric acid, dibasic sodium phosphate, glycerin and commercially available crystalline sorbitol polyethylene glycol 6000, polyvinylpyrrolidone, and N methyl 2-pyrrolidone

Procedure.—An absorption curve for acetylsalicylic acid and salicylic acid was prepared using buffer (pH 3 0) as the solvent Maximum absorption at this pH occurred at 275 mμ for acetylsalicylic acid and at 298 mμ for salicylic acid

Acetylsalicylic acid, 6 5 Gm portions, 100 mesh and 60 mesh, were accurately weighed into 125 ml glass-stoppered wide bottom flasks To each flask was added 100 ml of buffer solution composed of 3 *N* phosphoric acid adjusted to pH 3 0 with 3 *N* dibasic sodium phosphate This pH was chosen because it is the pH of a saturated acetylsalicylic acid solution At pH 3 0 the solubility of acetylsalicylic acid is approximately 4 22 Gm /L (4) Buffer solutions were added at the same temperatures used in the hydrolysis study

The flasks were immersed in a mechanical shaker constant temperature bath accurate to ±0 5° The hydrolysis rate was studied over a twenty four hour period at temperatures of 46, 50, and 60° by withdrawing aliquot portions of the supernatant liquid at zero time and at four hour intervals thereafter, using a sintered glass funnel attached to a pipet, diluting with buffer solution, and analyzing for

TABLE I —THE EFFECT OF TEMPERATURE ON THE HYDROLYSIS RATE OF ACETYLSALICYLIC ACID SUSPENSIONS[a]

	Mesh Size	Temperature ° C	[illegible]	$t_{1/2}$ Hr	ΔF Kcal /Mol	ΔHa KCal	ΔS Cal /Deg / Mole
A	60	25[b]		1169 0	5 204	18 472	44 52
	100			1200 0	5 221	18 872	44 80
B	60	46		[illegible]	4 474	18 472	43 88
	100				[illegible]470	18 872	45 14
C	60	50				18 472	44 52
	10[illegible]					18 872	45 81
D	6[illegible]					[illegible]8 472	45 64
	10[illegible]					[illegible]2	46 89
E	6[illegible]						28 89
F	6[illegible]						28 31
G	6[illegible]			[illegible]			28 88
H	60			7[illegible]			[illegible]06

A–D contain 6 [illegible]
[a] The ΔH [illegible]
[b] Values at 25 [illegible]

and the end products from moist aspirin anhydride, lot I, degradation at 70°, are identical (see Fig 9, A and B).

In addition, acetylsalicoylsalicylic acid (VII) was isolated from this pyrolyzed aspirin anhydride and identified.

A comparison of the spectra in chloroform solution of 2,2'-bis(2-acetoxybenzoyloxy)-benzoic anhydride (V) and aspirin anhydride (I), degraded at 70° for forty-eight hours, was almost identical The slight spectral difference in the degraded aspirin anhydride spectrum can be explained by the presence of a small amount of mixed anhydride such as the mixed anhydride of aspirin and acetic acid (II) (see Fig. 9, C and D).

The dashed line in Fig. 9D shows the spectra of the mixed anhydride of aspirin and acetic acid (II). It is readily seen that the additional high-frequency carbonyl band at 1,805 cm.$^{-1}$ in the degradation product can be attributed to intermediate mixed anhydrides The mixed anhydrides of aspirin-acetic acids and acetylsalicoylsalicylic-acetic acids have similar carbonyl frequencies in chloroform solution of 1,805, 1,758, 1,753, and 1,805, 1,740 cm.$^{-1}$, respectively. Both anhydrides can be represented by the dashed curve in Fig. 9D. The special drying of the aspirin anhydride prior to degradation reduced the possibility of hydrolytic decomposition, thus making it possible for the mixed anhydrides to persist longer than usual in this case. The relatively short decomposition time combined with ground "soft" glass catalysis allowed the existence of mixed anhydrides to be detected before their complete disproportionation to symmetrical anhydrides. A longer period of heating or a higher temperature would have led to complete destruction of the mixed anhydrides and the formation of the usual decomposition end products [2]

[2] This was shown by the fact that hydrolysis of the decomposition complex by boiling a 1-Gm sample with water for three hours resulted in a mixture of salicylic acid, aspirin, and acetylsalicoylsalicylic acid by I R analysis These products support the proposed mechanism of pyrolysis

SUMMARY

1. It is shown that dry aspirin anhydride in the absence of base at 40° and possibly also at 50° is stable indefinitely. The pyrolytic decomposition of aspirin anhydride is accelerated by alkalies or by impurities that will cause aspirin anhydride to melt and come in contact with basic surfaces, such as "soft" glass

2. It is definitely indicated that aspirin anhydride crystals are readily solvated, that solvation enhances pyrolytic degradation Micronization and subsequent drying favor increased stabilization to heat.

3. Pyrolytic degradation above 50° does not reflect case of degradation below 35° since aspirin anhydride pyrolysis has a very high heat of degradation, i. e., great changes in degradation rate with temperature

4. Infrared investigations of products, isolated and in the degrading melt, show the presence of all possible products of acyl transfer In moist systems the end products of the pyrolysis of aspirin anhydride are acetylsalicoylsalicylic acid and acetic acid In anhydrous systems the mixed and symmetrical anhydrides of these two acids are the major products

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The Stability of Acetylsalicylic Acid in Suspension*

By SEYMOUR M. BLAUG and JEREMII W. WESOLOWSKI

The effect of temperature and concentration on the hydrolysis rate of acetylsalicylic acid suspensions was studied. As would be expected for a zero-order reaction the more concentrated suspensions showed a longer half-life. The effect of additives on the stability of the aqueous suspensions was evaluated. Fifty per cent (w/v) crystalline sorbitol exerted the most pronounced stabilizing action. It raised the half-life of an aqueous suspension containing 6.5 Gm. of acetylsalicylic acid in 100 ml. of water from 1,748 hours to 3,396 hours at 25°.

THE IMMENSITY of the problem involved in preparing a liquid dosage form of acetylsalicylic acid can be summed up in the observation of Friedlander and Feinberg (1) ". . . Acetylsalicylic acid should never be used in aqueous solution because of its instability." Acetylsalicylic acid in aqueous media will hydrolyze almost completely in less than one week.

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Edwards (2, 3) made a thorough study of the kinetics of acetylsalicylic acid hydrolysis. He found the hydrolysis rate to be pseudo first order, the net velocity being a function of six possible reactions. The pH at which the greatest stability was obtained was between pH 2–3. This

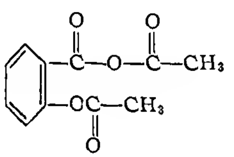
tion. However, Fig. 6 clearly shows that the carbonyl infrared of this large anhydride V is consistent with the postulate that the melt is a mixture of I and V. Comparison of the curve of Fig. 6 with those of Fig. 2 shows that V is consistent with the carbonyl spectra of partially degraded material.

The possibility of the existence of V as an intermediate in the degradation of moist aspirin anhydride is also consistent with the forms of the rate plots of degradation, particularly in Fig. 4. The spectra at 1,700 cm.$^{-1}$ continue to show large changes when that at 1,737.5 cm.$^{-1}$ (or even at 1,787.5 cm.$^{-1}$ in Fig. 3) shows small or none. This indicates the production of an intermediate, probably V, which is again transformed, probably by water to VII.

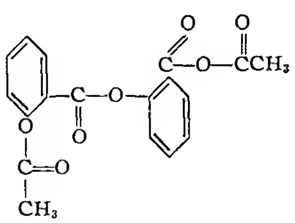
The degradation of the wetter lot I differed from lot II by showing a more definitive change in absorbance for 1,700 and 1,737.5 cm.$^{-1}$. The absorbance of lot II remained at much lower values than for lot I at these frequencies.

In a specific case where dry, pure aspirin anhydride (lot VII) was admixed with powdered soft glass and heated at 70°, liquefaction occurred readily. After a week, a chloroform solution of material showed a definite shoulder at 1,800 cm.$^{-1}$, a band of much higher frequency than in the carbonyl region of compounds I, V, and VII.

The possible derivative of aspirin anhydride with such a frequency was found to be the mixed anhydride of acetic acid and acetylsalicylic acid, II.

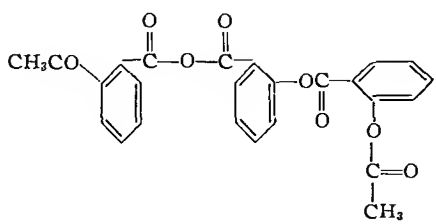


II



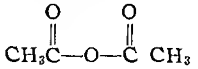
VI

It is also probable that the mixed anhydride of acetic acid and acetylsalicoylsalicylic acid VI would have a similar carbonyl spectrum and that the mixed anhydride of aspirin and acetylsalicoylsalicylic acid III would have a carbonyl spectrum similar to I and V.



III

Acetic anhydride (IV) is also a product of the pyrolytic degradation of aspirin anhydride.



IV

A mechanism for the pyrolytic degradation of aspirin anhydride consistent with the above facts would be acyl interchange with the ultimate products prior to hydrolyis, being acetic anhydride (IV) and the anhydride of acetylsalicoylsalicylic acid (V). A scheme which could represent all possible acyl exchanges is given in Fig. 10 where the solid lines represent the consequences of acyl exchange of the designated compound with aspirin anhydride, which should be in excess throughout the major part of the degradation. The two arrows from each compound represent the products of exchange. The reverse of the arrowed directions give the other possibilities, where one of the products would be aspirin anhydride and the other would be the compound at the origin of the arrows. The dashed lines represent the two products of acyl interchange of a compound with itself.

Of course, intramolecular acyl exchange is also possible with the monomolecular product of I being the mixed anhydride of acetic acid and acetylsalicoylsalicylic acid (VI).

The subsequent products of acyl exchange could be as diagrammed in Fig. 11. Two additional possible exchanges not covered by Figs. 10 and 11 are: VI + II $\longrightarrow$ III + IV and VI + III $\longrightarrow$ I + V.

The buildup of the anhydride of acetylsalicoylsalicylic acid (VI) and acetic anhydride (IV) as products of pyrolytic degradation can be readily concluded from these charts. The presence of extremely small amounts of water could yield acetylsalicoylsalicylic acid (VII) and acetic acid (VIII), since IV and V in the "melt" are readily hydrolyzed.

Further Evidence for Acetylsalicoylsalicylic Acid (VII) and 2,2'-Bis(2-acetoxybenzoyloxy)-benzoic Anhydride (V) as End Products of Aspirin Anhydride Pyrolysis.—The complete infrared spectra in chloroform solution of acetylsalicoylsalicylic acid (VII)

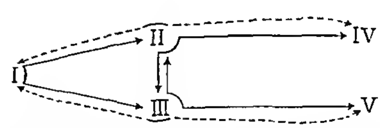


Fig. 10.—The products of acyl exchange. The solid arrows represent the two products of acyl exchange of a compound with aspirin anhydride. The dashed arrows represent the two products of acyl exchange of a compound with itself.

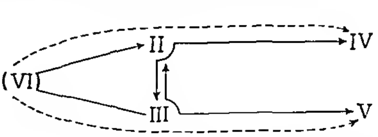


Fig. 11.—The products of acyl exchange from an initial intramolecular exchange, I → VI. The solid arrows represent the two products of acyl exchange of a compound with aspirin anhydride. The dashed arrows represent the two products of acyl exchange of a compound with itself.

and the end products from moist aspirin anhydride, lot I, degradation at 70°, are identical (see Fig. 9, A and B).

In addition, acetylsalicoylsalicylic acid (VII) was isolated from this pyrolyzed aspirin anhydride and identified.

A comparison of the spectra in chloroform solution of 2,2′-bis(2-acetoxybenzoyloxy)-benzoic anhydride (V) and aspirin anhydride (I), degraded at 70° for forty-eight hours, was almost identical. The slight spectral difference in the degraded aspirin anhydride spectrum can be explained by the presence of a small amount of mixed anhydride such as the mixed anhydride of aspirin and acetic acid (II) (see Fig. 9, C and D).

The dashed line in Fig. 9D shows the spectra of the mixed anhydride of aspirin and acetic acid (II). It is readily seen that the additional high-frequency carbonyl band at 1,805 cm.$^{-1}$ in the degradation product can be attributed to intermediate mixed anhydrides. The mixed anhydrides of aspirin-acetic acids and acetylsalicoylsalicylic-acetic acids have similar carbonyl frequencies in chloroform solution of 1,805, 1,758, 1,753, and 1,805, 1,740 cm.$^{-1}$, respectively. Both anhydrides can be represented by the dashed curve in Fig. 9D. The special drying of the aspirin anhydride prior to degradation reduced the possibility of hydrolytic decomposition, thus making it possible for the mixed anhydrides to persist longer than usual in this case. The relatively short decomposition time combined with ground "soft" glass catalysis allowed the existence of mixed anhydrides to be detected before their complete disproportionation to symmetrical anhydrides. A longer period of heating or a higher temperature would have led to complete destruction of the mixed anhydrides and the formation of the usual decomposition end products.[2]

[2] This was shown by the fact that hydrolysis of the decomposition complex by boiling a 1-Gm. sample with water for three hours resulted in a mixture of salicylic acid, aspirin, and acetylsalicoylsalicylic acid by I. R. analysis. These products support the proposed mechanism of pyrolysis.

SUMMARY

1. It is shown that dry aspirin anhydride in the absence of base at 40° and possibly also at 50° is stable indefinitely. The pyrolytic decomposition of aspirin anhydride is accelerated by alkalies or by impurities that will cause aspirin anhydride to melt and come in contact with basic surfaces, such as "soft" glass.

2. It is definitely indicated that aspirin anhydride crystals are readily solvated, that solvation enhances pyrolytic degradation. Micronization and subsequent drying favor increased stabilization to heat.

3. Pyrolytic degradation above 50° does not reflect ease of degradation below 35° since aspirin anhydride pyrolysis has a very high heat of degradation, i. e., great changes in degradation rate with temperature.

4. Infrared investigations of products, isolated and in the degrading melt, show the presence of all possible products of acyl transfer. In moist systems the end products of the pyrolysis of aspirin anhydride are acetylsalicoylsalicylic acid and acetic acid In anhydrous systems the mixed and symmetrical anhydrides of these two acids are the major products

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The effect of temperature and concentration on the hydrolysis rate of acetylsalicylic acid suspensions was studied. As would be expected for a zero-order reaction the more concentrated suspensions showed a longer half-life. The effect of additives on the stability of the aqueous suspensions was evaluated. Fifty per cent (w/v) crystalline sorbitol exerted the most pronounced stabilizing action. It raised the half-life of an aqueous suspension containing 6.5 Gm. of acetylsalicylic acid in 100 ml. of water from 1,748 hours to 3,396 hours at 25°.

THE IMMENSITY of the problem involved in preparing a liquid dosage form of acetylsalicylic acid can be summed up in the observation of Friedlander and Feinberg (1) "... *Acetylsalicylic acid* should never be used in aqueous solution because of its instability." Acetylsalicylic acid in aqueous media will hydrolyze almost completely in less than one week.

Edwards (2, 3) made a thorough study of the *kinetics of acetylsalicylic* acid hydrolysis. He found the hydrolysis rate to be pseudo first order, the net velocity being a function of six possible reactions. The pH at which the greatest stability was obtained was between pH 2–3. This

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represents the pH of a saturated acetylsalicylic acid solution

Garrett (4) extended Edwards' study to several acyl esters of salicylic acid in an attempt to correlate the interdependence of solubility and rate of degradation of saturated solutions If the degradation of homogenous solution is first order, the saturated solution should be zero order

The hydrolysis of acetylsalicylic acid depends upon the amount of acetylsalicylic acid in solution, hence suspensions of acetylsalicylic acid show a low degree of hydrolysis relative to the total amount of drug in suspension K C James (5) found that a 6 per cent unbuffered aqueous acetylsalicylic acid suspension, after storage for forty seven days at room temperature contained 89 per cent of the drug

Various techniques have been recommended for preparing a more stable acetylsalicylic acid solution Clark (6) developed a formula containing potassium citrate that will give a "minimum of hydrolysis" Organic solvents such as ethanol, propylene glycol, and glycerin solubilize the acid, but the disadvantage to their use lies in the fact that most patients will not find any of these solvents palatable Schwarz, *et al* (7), have developed an acetylsalicylic acid elixir which, according to the authors, holds promise as a pediatric preparation The formula recommended uses as solvent a blend of ethanol and polyethylene glycol 400 At the end of twenty four days, 17 7 per cent of the acetylsalicylic acid hydrolyzed in this solvent

A recent formulation (8) of an acetylsalicylic acid suspension has been found " to be stable for one month without excessive hydrolysis " According to the authors, crystalline sorbitol, when used in combination with other ingredients, exerted a pronounced stabilizing effect on the suspension Sorbitol has been used to stabilize vitamin C and B_{12} preparations (9), the action being attributed to two possible causes, the reduced availability of water in the sorbitol solution or complex formation between sorbitol and the drug

The objectives of this investigation were to study the kinetics of acetylsalicylic acid suspensions and the effects of various additives on the stability of the suspension

Higuchi, *et al* (10, 11), have reported on the interaction of acetylsalicylic acid and salicylic acid with several of the additives used in this study

EXPERIMENTAL

Reagents —Recrystallized acetylsalicylic acid, salicylic acid, calcium gluconate, reagent grade phosphoric acid, dibasic sodium phosphate, glycerin, and commercially available crystalline sorbitol, polyethylene glycol 6000, polyvinylpyrrolidone, and N methyl 2 pyrrolidone

Procedure.—An absorption curve for acetylsalicylic acid and salicylic acid was prepared using buffer (pH 3 0) as the solvent Maximum absorption at this pH occurred at 275 mμ for acetylsalicylic acid and at 298 mμ for salicylic acid

Acetylsalicylic acid, 6 5 Gm portions, 100 mesh and 60 mesh, were accurately weighed into 125 ml glass-stoppered wide bottom flasks To each flask was added 100 ml of buffer solution composed of 3 *N* phosphoric acid adjusted to pH 3 0 with 3 *N* dibasic sodium phosphate This pH was chosen because it is the pH of a saturated acetylsalicylic acid solution At pH 3 0 the solubility of acetylsalicylic acid is approximately 4 22 Gm /L (4) Buffer solutions were added at the same temperatures used in the hydrolysis study

The flasks were immersed in a mechanical shaker constant temperature bath accurate to ±0 5° The hydrolysis rate was studied over a twenty-four hour period at temperatures of 46, 50, and 60° by withdrawing aliquot portions of the supernatant liquid at zero time and at four hour intervals thereafter, using a sintered glass funnel attached to a pipet, diluting with buffer solution, and analyzing for

TABLE I —THE EFFECT OF TEMPERATURE ON THE HYDROLYSIS RATE OF ACETYLSALICYLIC ACID SUSPENSIONS[a]

	Mesh Size	Temperature ° C	$k \times 10^3$	$t_{1/2}$ Hr	ΔF Kcal /Mol	ΔHa KCal	ΔS Cal /Deg / Mole
A	60	25[b]	0 154	1169 0	5 204	18 472	44 52
	100		0 150	1200 0	5 221	18 872	44 80
B	60	46	0 867	207 6	4 474	18 472	43 88
	100		0 870	206 9	4 470	18 872	45 14
C	60	50	1 71	105 2	4 093	18 472	44 52
	100		1 76	102 2	4 075	18 872	45 81
D	60	60	7 16	25 1	3 273	18 472	45 64
	100		7 35	24 5	3 255	18 872	46 89
E	60	25[b]	0 273	1321 4	4 863	13 472	28 89
F	60	46	0 913	395 1	4 441	13 472	28 31
G	60	50	1 58	228 3	4 144	13 472	28 88
H	60	60	5 37	67 2	3 462	13 472	30 06

A–D contain 6 5 Gm acetylsalicylic acid/100 ml E–H contain 13 0 Gm acetylsalicylic acid/100 ml

[a] The ΔH ionization for water was calculated to be 12 928 Kcal in the temperature range studied

[b] Values at 25° calculated from knowledge of ΔHa

salicylic acid spectrophotometrically at 298 mμ. The same procedure was repeated on suspensions containing 13.0 Gm. of 60-mesh acetylsalicylic acid. All suspensions were prepared in duplicate. Results are shown in Table I and in Figs. 1, 2, and 3. The thermodynamic values reported in Table I were obtained from plotting log k *vs.* $1/T$ and calculated from the following equations:

$$(a) \qquad \Delta F = -RT \ln K$$

$$(b) \qquad \frac{\Delta F - \Delta H}{T} = -\Delta S$$

The effect of additives on the stability of acetylsalicylic acid suspensions was evaluated by preparing varying per cent solutions of the agents under investigation in the buffer solution. Glycerin and sorbitol were also prepared in distilled water in order to explain more fully the reported (8) stabilizing action of sorbitol on acetylsalicylic acid suspensions.

Acetylsalicylic acid, 6.5-Gm. portions, 60-mesh, were accurately weighed into 125-ml. glass-stoppered wide-bottom flasks. To each flask was added 100 ml. of solution of the agent under investigation. Solutions were added at 50° and the flasks were immersed in a mechanical shaker constant temperature bath adjusted to 50° (±0.5°). All suspensions were prepared in duplicate.

Aliquot portions of the supernatant liquid were removed at zero time and at four-hour intervals thereafter and diluted with buffer solution to a volume which enabled spectrophotometric determination of salicylic acid. Samples were taken over a twenty-four-hour period. Agents studied and results are shown in Table II and in Fig. 4.

TABLE II.—EFFECT OF ADDITIVES ON THE HYDROLYSIS OF ACETYLSALICYLIC ACID SUSPENSIONS[a]

Compound	Concentration, (w/v) %	$k \times 10^{3c}$	$t^{1/2}$ Hr.[d]
Distilled water	..	1.21	149
Buffer pH 3.0	..	1.76	102
Calcium gluconate[b]	1	1.93	93.3
Glycerin	50	3.42	52.6
Glycerin[b]	50	1.32	136.5
N-Methyl-2-pyrrolidone	4	1.78	90
Polyethylene glycol 6000	3	0.385	468
Polyethylene glycol 6000	2	0.477	378
Polyethylene glycol 6000	1	1.0	180
Polyvinylpyrrolidone	1	0.435	414
Polyvinylpyrrolidone	0.25	0.98	204
Polyvinylpyrrolidone	0.1	1.27	142
Polyvinylpyrrolidone }	1	..	..
N-Methyl-2-pyrrolidone }	4	0.60	300
Polyvinylpyrrolidone }	1	..	..
N-Methyl-2-pyrrolidone }	2	0.57	316
Salicylic acid	0.01	1.74	103.5
Sorbitol, crystalline	50	1.36	132.5
Sorbitol, crystalline[b]	50	0.625	288

[a] 6.5% suspensions of 100-mesh acetylsalicylic acid prepared in pH 3.0 buffer at 50° except where otherwise noted.
[b] Prepared in distilled water.
[c] Obtained from a plot of acetylsalicylic acid concentration *vs.* time.
[d] $t^{1/2} = a/2k$ (a = initial acetylsalicylic acid concentration).

DISCUSSION

Acetylsalicylic acid suspensions prepared in an aqueous solvent exhibited a longer half-life than the corresponding suspension prepared in a pH 3.0

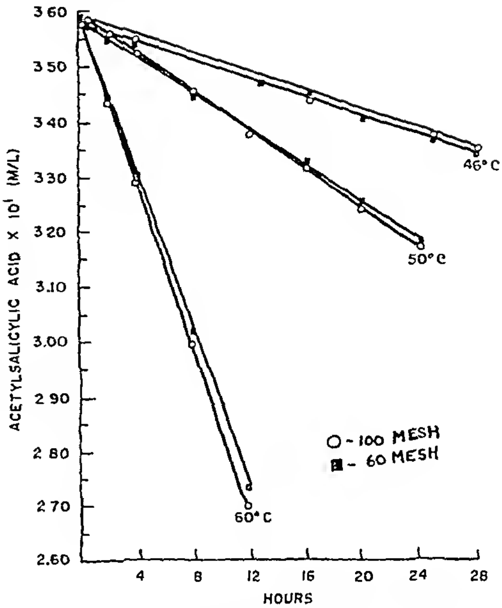

Fig. 1.—Hydrolysis of acetylsalicylic acid suspension (6.5% w/v).

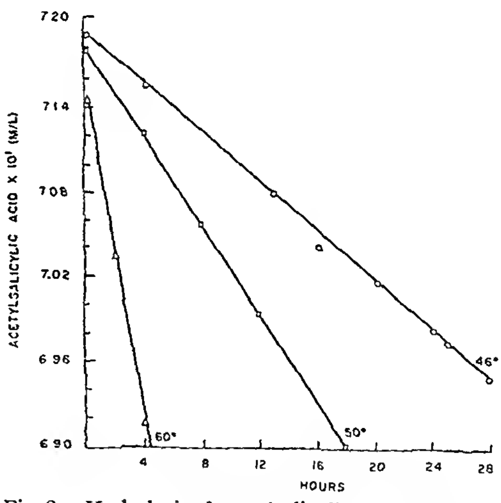

Fig. 2.—Hydrolysis of acetylsalicylic acid suspension (13% w/v).

buffer. This was due to the lower final pH (2.5) of the unbuffered suspension.

It was found that the polyethylene glycol 6000 and polyvinylpyrrolidone exhibited apparently dramatic stabilizing effects on acetylsalicylic acid in suspension. However, on closer investigation it was found that an insoluble gummy precipitate formed which was very difficult to redisperse. Previous workers (10, 11) reported that P. E. G. 6000 and polyvinylpyrrolidone formed soluble complexes with salicylic acid at 30°. At the temperature used in this study the complex formed probably has a lower solubility than it does at 30°. This will be verified in another study now under way. Therefore, the hydrolysis constants obtained are only indicative of the salicylic acid not present in the insoluble complex.

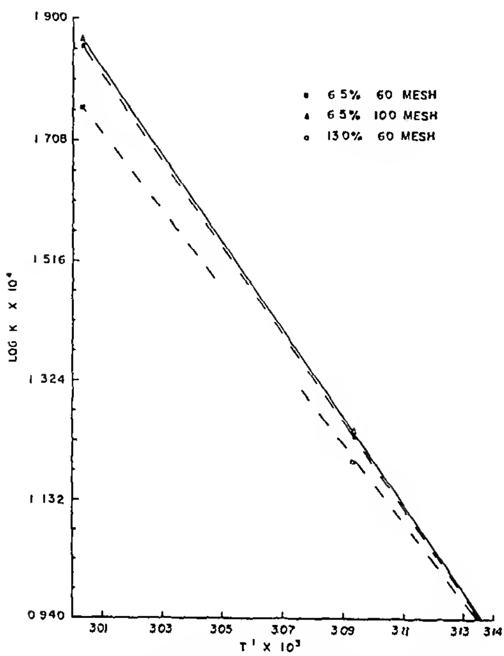

Fig 3 —The effect of temperature on the hydrolysis rate of acetylsalicylic acid suspensions

N-Methyl-2-pyrrolidone enhanced the solubility of acetylsalicylic acid, therefore an increase in hydrolysis rate was observed

Calcium gluconate accelerated the hydrolysis of acetylsalicylic acid in suspension because of the high pH of the aqueous solution This was contrary to the reported (12) stabilizing action of calcium gluconate on aqueous acetylsalicylic acid preparations.

Acetylsalicylic acid suspensions were prepared in which the supernatant liquid was saturated with salicylic acid. This procedure was without effect on the hydrolysis rate

The effect of glycerin was studied in aqueous and buffered suspensions In both cases glycerin was found to increase the hydrolysis rate to a significant degree Since the hydrolysis rate is directly proportional to the concentration of acetylsalicylic acid in solution, the increased hydrolysis rate was probably due to the better solvent action of the glycerin-water solution.

Fifty per cent crystalline sorbitol in aqueous acetylsalicylic acid suspension exerted a pronounced stabilizing action The half-life was raised from 1,748 hours to 3,396 hours at 25°. Even in the buffered suspension there was some stabilizing action but it was not as great as in the unbuffered suspension. A separate investigation is being conducted on the action of sorbitol on the stability of several medicaments

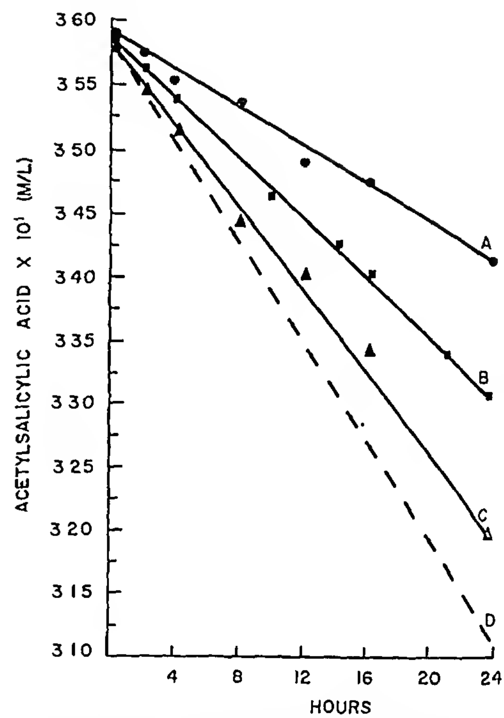

Fig. 4 —Hydrolysis of acetylsalicylic acid suspension at 50° in: A, 50% sorbitol (w/v) in water B, water alone C, 50% sorbitol (w/v) in pH 3 0 buffer. D, pH 3 0 buffer alone

As would be expected for a zero-order reaction the more concentrated suspension showed a longer half-life Since the hydrolysis rate is independent of the total acetylsalicylic acid concentration and dependent on the amount of acetylsalicylic acid in solution, the acetylsalicylic acid hydrolysis would be smaller in proportion to the total acetylsalicylic acid concentration, for the more concentrated suspension.

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Steroidal Sapogenins LV*

Survey of Plants for Steroidal Sapogenins and Other Constituents

By MONROE E. WALL, C. S. FENSKE, J. W. GARVIN, J. J. WILLAMAN, QUENTIN JONES, BERNICE G. SCHUBERT, and H. S. GENTRY

This report covers the fifth 1,000 accessions in a survey of plants for steroidal sapogenins and is thus a continuation of the previous four reports (1–7). Data are given for 990 accessions (all but 107 from the United States), representing 147 families, 547 genera, and 921 species. Few steroidal sapogenins were found, largely because of the absence of *Agave, Yucca,* and *Dioscorea;* but alkaloids were qualitatively identified in 86 species, 45 of which are new to the record.

THIS IS A CONTINUATION of the previous reports (1–7) on the first four 1,000 accessions. It covers the fifth 1,000 plant samples in our survey of the plant world for steroidal sapogenins, and includes qualitative results for flavonoids, alkaloids, tannins, and unsaturated sterols.

PROCUREMENT

By 1954 the search for plant sources of steroidal sapogenins by the three cooperating federal agencies had been in operation for approximately four years. Leads developed during this period indicated that the three genera, *Dioscorea, Agave,* and *Yucca,* were the most promising sources of useful steroidal sapogenins. Procurement activities in the latter part of this period reflected the attention focused on these genera and their near relatives. Representatives of many other families were examined but the major emphasis became centered on the *Dioscoreaceae, Amaryllidaceae,* and *Liliaceae.*

The present report chronicles a different phase of of the program. Species of *Dioscorea* had been settled upon as having the most potential for becoming crop sources of cortical and hormonal analogs. Intensive crop development studies by the Crops Research Division, guided in the selection of superior strains by chemical evaluations supplied by the Eastern Utilization Research and Development Division, had naturally become an increasingly important phase of the program. But because *Dioscorea, Agave,* and *Yucca* had by this time been rather thoroughly screened for steroidal sapogenins, procurement activities were directed once more toward a broad survey of the higher plants with the hope of finding a steroid-rich species more amenable to crop conditions of the United States than are species of *Dioscorea.*

The logical place to look for species which are apt to fit crop environments of this country is in our native flora. Of the 990 accessions here reported on, all but 107 were collected in the United States. Collecting was concentrated in the Atlantic States as evidenced by over 800 accessions here listed from that area.

Another reflection of the broadness of the screening program during this period is the large number of families, genera, and species represented. Included are 921 species in 547 genera distributed through 147 families. This same philosophy of obtaining broad coverage of our native flora continues with other areas of the country receiving increasingly more emphasis in our procurement activities.

REVISED ALKALOID PROCEDURE

In previous reports we were conducting the Mayer alkaloid tests on samples in which each ml. of final test solution was equivalent to 0.2 Gm. of original dry plant sample. Because we were obtaining a much larger proportion of negative alkaloid tests than expected from statistical averages, we have greatly increased the concentrations used so that 1 ml. of final test solution is equivalent to 4.0 Gm. of dry plant material. Thus the revised procedure is much more sensitive for the purposes of alkaloid screening.

RESULTS

A compilation of all data is given in Table I, page 696, *et seq.* Since in the past steroidal sapogenins were found mostly in *Agave, Yucca,* and *Dioscorea,* and since in the present collection these genera were represented by only six collections, the table, as previously used, has been modified by the omission of the columns concerning sapogenins. Their presence is indicated by footnote *b* and the details are reported in the text.

Saponins, as indicated by hemolysis, were found in only 10% of the species. This is a surprisingly low incidence, since in the previous 4,000 accessions the occurrence was 35% of the species exclusive of those in *Agave, Dioscorea,* and *Yucca.* The following list gives the information on the steroidal sapogenins found; the accession number, the plant, the plant part, the amount, and kind of sapogenins: 4127, 4705 *Dioscorea deltoidea* (*Dioscoreaceae*); r, t; 0.5 and 1.2%; diosgenin. 4138 *Dioscorea quaternata;* r; 0.5%; diosgenin. 4653 *Clintonia umbellulata* (*Liliaceae*); l, s, r; 0.1%; not determined. 4678 *Trillium flexipes* (*Liliaceae*); l, s, fr; 0.3%; not identified. 4713B *Cestrum* sp. (*Solanaceae*); fr; 0.5%; tigogenin. 4743 *Tribulus terrestris* (*Zygophyllaceae*); l, s, fl; 0.3%; tigogenin.

* Received August 4, 1959, from M. E. Wall, C. S Fenske, J. W. Garvin, and J. J. Willaman of Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Dept. of Agriculture, Philadelphia 18, Pa., and Quentin Jones, Bernice G. Schubert, and H. S. Gentry of New Crops Research Branch, Crops Research Division, Agricultural Research Service, U. S. Dept of Agriculture, Beltsville, Md.

A substantial part of the cost of publication of this survey was contributed by the United States Department of Agriculture.

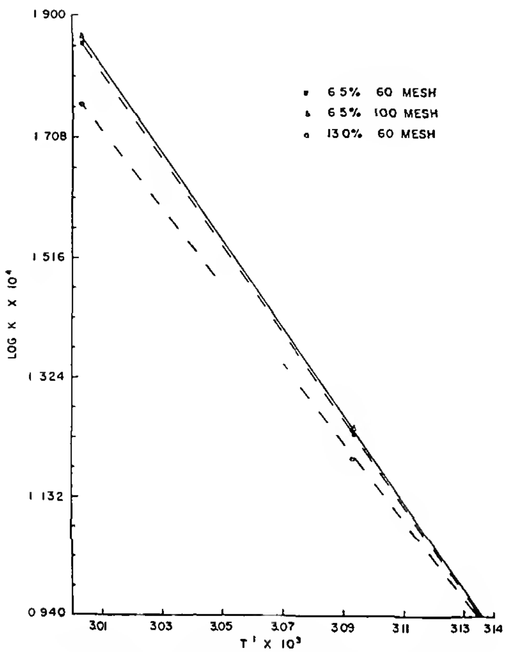

Fig 3 —The effect of temperature on the hydrolysis rate of acetylsalicylic acid suspensions

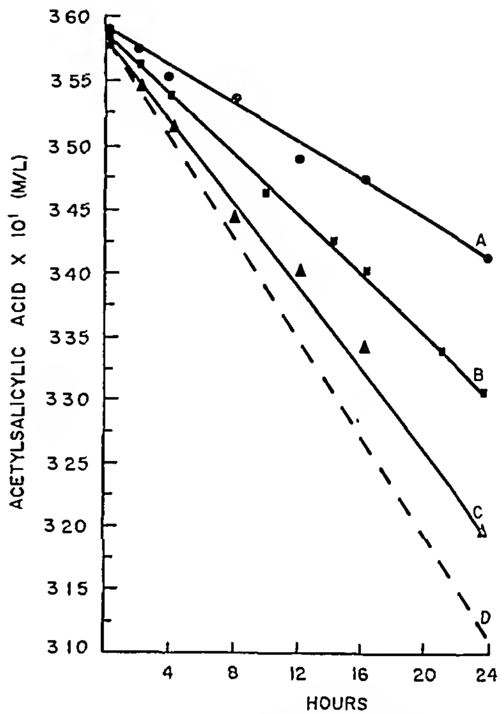

Fig 4 —Hydrolysis of acetylsalicylic acid suspension at 50° in: A, 50% sorbitol (w/v) in water. B, water alone C, 50% sorbitol (w/v) in pH 3.0 buffer. D, pH 3.0 buffer alone.

N-Methyl-2-pyrrolidone enhanced the solubility of acetylsalicylic acid, therefore an increase in hydrolysis rate was observed.

Calcium gluconate accelerated the hydrolysis of acetylsalicylic acid in suspension because of the high pH of the aqueous solution. This was contrary to the reported (12) stabilizing action of calcium gluconate on aqueous acetylsalicylic acid preparations.

Acetylsalicylic acid suspensions were prepared in which the supernatant liquid was saturated with salicylic acid. This procedure was without effect on the hydrolysis rate

The effect of glycerin was studied in aqueous and buffered suspensions In both cases glycerin was found to increase the hydrolysis rate to a significant degree Since the hydrolysis rate is directly proportional to the concentration of acetylsalicylic acid in solution, the increased hydrolysis rate was probably due to the better solvent action of the glycerin-water solution.

Fifty per cent crystalline sorbitol in aqueous acetylsalicylic acid suspension exerted a pronounced stabilizing action The half-life was raised from 1,748 hours to 3,396 hours at 25°. Even in the buffered suspension there was some stabilizing action but it was not as great as in the unbuffered suspension A separate investigation is being conducted on the action of sorbitol on the stability of several medicaments

As would be expected for a zero-order reaction the more concentrated suspension showed a longer half-life Since the hydrolysis rate is independent of the total acetylsalicylic acid concentration and dependent on the amount of acetylsalicylic acid in solution, the acetylsalicylic acid hydrolysis would be smaller in proportion to the total acetylsalicylic acid concentration, for the more concentrated suspension.

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Steroidal Sapogenins LV*

Survey of Plants for Steroidal Sapogenins and Other Constituents

By MONROE E. WALL, C. S. FENSKE, J. W. GARVIN, J. J. WILLAMAN, QUENTIN JONES, BERNICE G. SCHUBERT, and H. S. GENTRY

This report covers the fifth 1,000 accessions in a survey of plants for steroidal sapogenins and is thus a continuation of the previous four reports (1–7). Data are given for 990 accessions (all but 107 from the United States), representing 147 families, 547 genera, and 921 species. Few steroidal sapogenins were found, largely because of the absence of *Agave, Yucca,* and *Dioscorea;* but alkaloids were qualitatively identified in 86 species, 45 of which are new to the record.

THIS IS A CONTINUATION of the previous reports (1–7) on the first four 1,000 accessions. It covers the fifth 1,000 plant samples in our survey of the plant world for steroidal sapogenins, and includes qualitative results for flavonoids, alkaloids, tannins, and unsaturated sterols.

PROCUREMENT

By 1954 the search for plant sources of steroidal sapogenins by the three cooperating federal agencies had been in operation for approximately four years. Leads developed during this period indicated that the three genera, *Dioscorea, Agave,* and *Yucca,* were the most promising sources of useful steroidal sapogenins. Procurement activities in the latter part of this period reflected the attention focused on these genera and their near relatives. Representatives of many other families were examined but the major emphasis became centered on the *Dioscoreaceae, Amaryllidaceae,* and *Liliaceae.*

The present report chronicles a different phase of of the program. Species of *Dioscorea* had been settled upon as having the most potential for becoming crop sources of cortical and hormonal analogs. Intensive crop development studies by the Crops Research Division, guided in the selection of superior strains by chemical evaluations supplied by the Eastern Utilization Research and Development Division, had naturally become an increasingly important phase of the program. But because *Dioscorea, Agave,* and *Yucca* had by this time been rather thoroughly screened for steroidal sapogenins, procurement activities were directed once more toward a broad survey of the higher plants with the hope of finding a steroid-rich species more amenable to crop conditions of the United States than are species of *Dioscorea.*

The logical place to look for species which are apt to fit crop environments of this country is in our native flora. Of the 990 accessions here reported on, all but 107 were collected in the United States. Collecting was concentrated in the Atlantic States as evidenced by over 800 accessions here listed from that area.

Another reflection of the broadness of the screening program during this period is the large number of families, genera, and species represented. Included are 921 species in 547 genera distributed through 147 families. This same philosophy of obtaining broad coverage of our native flora continues with other areas of the country receiving increasingly more emphasis in our procurement activities.

REVISED ALKALOID PROCEDURE

In previous reports we were conducting the Mayer alkaloid tests on samples in which each ml of final test solution was equivalent to 0.2 Gm. of original dry plant sample. Because we were obtaining a much larger proportion of negative alkaloid tests than expected from statistical averages, we have greatly increased the concentrations used so that 1 ml. of final test solution is equivalent to 4.0 Gm. of dry plant material. Thus the revised procedure is much more sensitive for the purposes of alkaloid screening.

RESULTS

A compilation of all data is given in Table I, page 696, *et seq.* Since in the past steroidal sapogenins were found mostly in *Agave, Yucca,* and *Dioscorea,* and since in the present collection these genera were represented by only six collections, the table, as previously used, has been modified by the omission of the columns concerning sapogenins. Their presence is indicated by footnote *b* and the details are reported in the text.

Saponins, as indicated by hemolysis, were found in only 10% of the species. This is a surprisingly low incidence, since in the previous 4,000 accessions the occurrence was 35% of the species exclusive of those in *Agave, Dioscorea,* and *Yucca.* The following list gives the information on the steroidal sapogenins found; the accession number, the plant, the plant part, the amount, and kind of sapogenins: 4127, 4705 *Dioscorea deltoidea* (*Dioscoreaceae*); r, t; 0.5 and 1.2%; diosgenin. 4138 *Dioscorea quaternata;* r; 0.5%; diosgenin. 4653 *Clintonia umbellulata* (*Liliaceae*); l, s, r; 0.1%; not determined. 4678 *Trillium flexipes* (*Liliaceae*); l, s, fr; 0 3%; not identified. 4713B *Cestrum* sp. (*Solanaceae*); fr; 0.5%; tigogenin. 4743 *Tribulus terrestris* (*Zygophyllaceae*); l, s, fl; 0.3%; tigogenin.

* Received August 4, 1959, from M. E. Wall, C S Fenske, J. W. Garvin, and J J Willaman of Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S Dept. of Agriculture, Philadelphia 18, Pa, and Quentin Jones, Bernice G Schubert, and H. S Gentry of New Crops Research Branch, Crops Research Division, Agricultural Research Service, U. S Dept of Agriculture, Beltsville, Md.

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TABLE I.—CONSTITUENTS FOUND IN THE PLANT COLLECTIONS

Code for Source Names

C. G. F.—U. S. Plant Introduction Garden, Coconut Grove, Fla. (cult.)
Chico, Calif.—U. S. Plant Introduction Garden, Chico, Calif. (cult.)

Code for Plant Parts

b—bark	r—root
bu—bulb	s—stem
fl—influorescence	t—tuber
fr—fruit	tw—twig
l—leaf	w—whole plant above ground

Accession No.	Species (Collection)	Source (Collection)	Date Collected (Collection)	Plant Part (Collection)	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			ACANTHACEAE						
4028	*Aphelandra nitens*	Tingo Maria, Peru	4/54	l, s, r	+[a]	0	0	0	+++
4253	*Dianthera (Justicia) Americana*	Johnston Co., N. C.	6/54	l, s	−	0	0	0	0
			ACERACEAE						
4252	*Acer Floridanum*	Wake Co., N. C.	6/54	l, s	−	0	0	+	0
4510	*Acer Pensylvanicum*	Haywood Co., N. C.	7/54	l, s, fr	−	+	0	+++	..
			AIZOACEAE						
4588	*Mollugo verticillata*	Brunswick, N. C.	7/54	l, s, r	+[a]	0	0	0	..
4680	*Mollugo verticillata*	Dorchester Co., Md.	7/54	l, s, fl, r	−	0	0	++	..
4897	*Sesuvium portulacastrum*	Franklin Co., Fla.	8/54	l, s	−	0	0	0	..
			ALISMACEAE						
4689	*Alisma subcordatum*	Dorchester Co., Md.	7/54	l, s, fl, r	−	0	0	0	..
4439	*Lophotocarpus calycinus*	Brunswick Co., N. C.	7/54	l, s, fr, r	−	0	0	0	..
4847	*Sagittaria engelmanniana*	Wayne Co., Pa.	8/54	l, s, fl, r	−	0	0	0	..
4744	*Sagittaria falcata*	Carteret Co., N. C.	7/54	l, s	−	0	0	0	..
4668	*Sagittaria latifolia*	Baltimore Co., Md.	7/54	l, s, r	−	0	0	0	..
			AMARANTHACEAE						
4747	*Acnida cannabina*	Carteret Co., N. C.	7/54	l, s	+[a]	0	0	0	..
4688	*Acnida cannabina*	Dorchester Co., Md.	7/54	l, s, r	−	0	0	0	..
4197	*Alternanthera philoxeroides*	New Hanover Co., N. C.	6/54	l, s, fl, r	+[a]	0	0	0	++
4682	*Amaranthus graecizans*	Dorchester Co., Md.	7/54	l, s, fl, r	−	0	0	0	..
4478	*Amaranthus hybridus*	Nansemond Co., Va.	7/54	l, s, r	+[a]	0	0	0	..
4732	*Amaranthus spinosus*	New Hanover Co., N. C.	7/54	l, s, fl, r	+[a]	0	0	0	..
4892	*Froelichia Floridana*	Leon Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
			ANACARDIACEAE						
4153	*Pistacia lentiscus*	Chico, Calif.	5/54	l, s	−	+++	0	+++	+
4051	*Rhus aromatica*	Hancock Co., Ga.	4/54	l	−	0	0	+	+
4073	*Rhus copallina*	McIntosh Co., Ga.	4/54	l	−	0	0	+++	++
4082	*Rhus glabra*	Clarke Co., Ga.	4/54	l	−	0	0	++	0
4525	*Rhus glabra*	Buncombe Co., N. C.	7/54	s, fr	−	0	0	+++	..
4787	*Rhus ovata*	Aguanga, Calif.	7/54	l	−	0	0	+++	..
4659	*Rhus typhina*	Garrett Co., Md.	7/54	l, s, fl	−	0	0	+++	..
4286	*Rhus vernix*	Anne Arundel Co., Md.	6/54		−	0	0	0	..

			ANNONACEAE						
1386	*Annona cherimola*	C G F	7/54	l, s	—	0	++	0	
1716	*Annona glabra*	C G F	7/54	l, s, fr	—	0	++	0	
4717	*Annona reticulata*	C G F	7/54	l, s	—	0	++	0	
1317	*Asimina pygmaea*	Leon Co, Fla	6/54	l, s, fr	—	0	0	0	
4221	*Asimina triloba*	Wake Co, N C	6/54	l, s	—	0	0	0	0
			APOCYNACEAE						
1377	*Adenium obesum*	C G F	7/54	l, s	—	0	0	0	
1712	*Alamanda violacea*	C G F	7/54	l, s	—	0	0	0	
4052	*Amsonia ciliata*	Johnson Co, Ga	4/54	l	—	0	+	0	++
1828	*Apocynum androsaemifolium*	Cayuta Lake, N Y	8/54	l, s, fl, r	—	0	0	0	
1065	*Apocynum cannabinum*	McIntosh Co, Ga	4/54	l	—	0	0	0	++
			AQUIFOLIACEAE						
4153	*Ilex cassine*	Brunswick Co, N C	7/54	l, s, fr	—	0	+	0	
1265	*Ilex vomitoria*	Brunswick Co, N C	6/54	l, s, r	—	0	0	0	
			ARACEAE						
1216	*Arisaema triphyllum*	Wake Co, N C	6/54	l, s, fr, bu	—	0	0	0	0
1011	*Colocasia sp*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	+
4102	*Orontium aquaticum*	Atlantic Co, N J	7/54	l, s, fl, r	—	0	0	0	
1715	*Peltandra Virginica*	Carteret Co, N C	7/54	l, s, fr	—	0	0	0	
4151	*Symplocarpus foetidus*	Grant Co, W Va	5/54	l, s, r	+[a]	0	0	0	+
			ARALIACEAE						
4656	*Aralia nudicaulis*	Monongalia Co, W Va	7/54	l, s, fr	—	0	0	0	
1561	*Aralia racemosa*	Macon Co, N C	7/54	l, s, r	—	0	0	0	
4605	*Aralia racemosa*	Clay Co, W Va	7/54	l, s, fl, r	—	0	0	0	
1304	*Aralia spinosa*	Lancaster Co, Va	6/54	l, s	—	0	0	0	
			ARISTOLOCHIACEAE						
1596	*Aristolochia durior*	Haywood Co, N C	7/54	l, s	—	0	0	0	
1604	*Aristolochia durior*	Clay Co, W Va	7/54	l, s, fr	—	0	0	0	
1118	*Asarum canadense*	Preston Co, W Va	5/54	l, s, r	—	0	+	0	+
4220	*Asarum reflexum*	Wake Co, N C	6/54	l s, r	—	0	0	0	0
			ASCLEPIADACEAE						
1628	*Ambelamus albidus*	Union Co, Ky	7/54	l, s, fl, r	—	0	0	0	
1334	*Asclepias amplexicaulis*	Wake Co, N C	6/54	l, s, fr, r	+[a]	0	0	0	
1799	*Asclepias Californica*	Dripping Springs, Calif	7/54	w	+[a]	0	0	0	
1079	*Asclepias humistrata*	Bryan Co, Ga	4/54	l	—	0	0	0	++
1685	*Asclepias incarnata*	Dorchester Co, Md	7/54	l, s, fl, r	—	0	0	0	
4901	*Asclepias cf obovata*	Franklin Co, Fla	8/54	l, s, fl, r	—	0	0	0	
1190	*Asclepias syriaca*	Nansemond Co, Va	7/54	l, s, fr	—	0	0	0	
1245	*Asclepias tuberosa*	Johnston Co, N C	6/54	l, s, fl, r	—	0	0	0	+++
1928	*Asclepias verticillata*	Charles Co, Md	8/54	l, s, r	—	0	0	0	
1777	*Asclepias (Acerates) viridiflora*	Baltimore Co, Fla	7/54	l, s, fr, r	—	+	0	0	
1750	*Gonolobus suberosus*	Carteret Co, N C	7/54	l, s, fr	—	0	0	0	
			BALSAMINACEAE						
1247	*Impatiens capensis (I biflora)*	Wake Co, N C	6/54	l, s	+[a]	0	0	0	+

Accession No	Species	Collection Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
		BEGONIACEAE							
4008	*Begonia foliosa* var *Australis*	Cayumba, Tingo Maria, Peru	4/54	l, s, r	—	0	0	0	0
		BERBERIDACEAE							
4530	*Akebia quinata*	Buncombe Co, N C	7/54	l, s	—	0	0	0	
4546	*Berberis thunbergii*	Henderson Co, N C	7/54	l, s	—	0	+++	0	
4529	*Caulophyllum thalictroides*	Buncombe Co, N C	7/54	l, s, fr, r	+[a]	0	++	0	
1551	*Diphylleia cymosa*	Macon Co, N C	7/54	l, s, fr	—	+++	0	0	
		BETULACEAE							
4507	*Betula lenta*	Haywood Co, N C	7/54	l, s	—	0	0	+	
1083	*Betula nigra*	Clarke Co, Ga	4/54	l	+[a]	0	0	0	0
4845	*Betula populifolia*	Wayne Co, Pa	8/54	l, s	—	0	0	++	
4325	*Carpinus Caroliniana*	Chatham Co, N C	6/54	l, s	—	0	0	0	
4393	*Corylus Americana*	Baltimore Co, Md	7/54	l, s	—	0	0	0	
4526	*Ostrya Virginiana*	Buncombe Co, N C	7/54	l, s	—	+	0	0	
4666	*Ostrya Virginiana*	Allegany Co, Md	7/54	l, s	—	0	0	0	
		BIGNONIACEAE							
4719	*Arrabidaea rotundata*	C G F	7/54	l, s	—	0	0	0	
4611	*Bignonia capreolata*	Floyd Co, Ky	7/54	l, s	+[a]	0	0	0	
4365	*Catalpa speciosa*	Jefferson Co, Ga	6/54	l, s	—	0	0	0	
4089	*Campsis radicans*	Clarke Co, Ga	4/54	l, s	—	0	0	0	+++
4923	*Campsis radicans*	Charles Co, Md	8/54	fr	—	0	0	0	
		BIXACEAE							
4009	*Bixa orellana*	Cayumba, Tingo Maria, Peru	4/54	l, s	—	0	0	+++	++
		BORAGINACEAE							
4025	*Cordia sp*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	0
4806	*Cynoglossum officinale*	Yates Co, N Y	8/54	l, s, fl, r	—	0	0	0	
4102	*Cynoglossum Virginianum*	Wake Co, N C	4/54	l, r	—	0	0	0	0
4204	*Echium vulgare*	Wake Co, N C	6/54	l, s, fl	—	0	0	0	0
4501	*Heliotropium anchusaefolium*	Marlboro Co, S C	7/54	l, s, fl	—	0	+	0	
4638	*Heliotropium tenellum*	Bullitt Co, Ky	7/54	l	+[a]	0	0	0	
		BROMELIACEAE							
4756	*Tillandsia usneoides*	Carteret Co, N C.	7/54	l, fl	—	+	0	0	
		BUXACEAE							
4615	*Pachysandra procumbens*	Laurel Co, Ky	7/54	l, s, r	—	0	0	0	
		CACTACEAE							
4295	*Opuntia humifusa*	Anne Arundel Co, Md	6/54	w	—	0	0	0	
		CAMPANULACEAE							
4652	*Campanula Americana*	Gilmer Co, W Va	7/54	l, s, fl, r	—	0	0	0	
4808	*Campanula rapunculoides*	Yates Co, N Y	7/54	l, s, fl, r	—	0	0	0	
4292	*Specularia perfoliata*	Charles Co, Md	6/54	l, s, fl, r	—	0	0	0	
4056	*Specularia perfoliata*	McIntosh Co, Ga.	4/54	l	—	0	0	0	

			CANNACEAE						
4763	*Canna flaccida*	Carteret Co., N. C.	7/54	l, s, fr, r	−	0	0	0	..
			CAPPARIDACEAE						
4786	*Cleome spinosa*	Baltimore Co., Md.	7/54	l, s, r	+[a]	0	0	0	..
4835	*Polanisia graveolens*	Mecklenberg, N. Y.	8/54	l, s, fr, r	−	+	+	0	..
			CAPRIFOLIACEAE						
4881	*Abelia grandiflora* (cult.)	Leon Co., Fla.	8/54	l, s, fl	−	0	0	0	..
4810	*Diervilla lonicera*	Yates Co., N. Y.	8/54	l, s, fr, r	−	0	0	0	..
4552	*Diervilla sessilifolia*	Macon Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4249	*Lonicera sempervirens*	Wake Co., N. C.	6/54	l, s	−	0	0	0	..
4640	*Symphoricarpus orbiculatus*	Bullitt Co., Ky.	7/54	l, s, fl, r	−	0	0	0	..
4853	*Viburnum acerifolium*	Federick Co., Md.	8/54	l, s, r	−	0	0	++	..
4842	*Viburnum cassinoides*	Wayne Co., Pa	8/54	l, s, fr	−	0	0	0	..
4831	*Viburnum dentatum*	Cayuta Lake, N. Y.	8/54	l, s	−	0	0	+	..
4830	*Viburnum opulus*	Yates Co., N. Y.	8/54	l, s, fr	−	0	0	0	..
4092	*Viburnum rufidulum*	Clarke Co., Ga.	4/54	l	−	0	0	0	++
4829	*Viburnum sp.*	Cayuta Lake, N. Y.	8/54	l, s	−	0	0	0	..
			CARYOPHLLACEAE						
4078	*Arenaria Caroliniana*	Bryan Co., Ga.	4/54	l	+[a]	0	0	0	0
4693	*Dianthus armeria*	Accomac, Va.	7/54	l, s, fl, r	+[a]	0	0	0	..
4895	*Odontonychia erecta*	Franklin Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
4608	*Paronychia Canadensis*	Wyoming Co., W. Va.	7/54	l, s, r	−	0	0	++	..
4883	*Paronychia fastigiata*	Leon Co., Fla.	8/54	l, s, fl	−	0	0	0	..
4983	*Paronychia herniarioides*	Liberty Co., Fla.	8/54	l, s, fl	−	0	0	++	..
4090	*Saponaria officinalis*	Clarke Co., Ga.	4/54	l	+[a]	0	0	0	+
4303	*Scleranthus annuus*	Anne Arundel Co., Md.	6/54	l, s	−	0	0	0	..
4128	*Silene Caroliniana* var. *Pensylvanica*	Washington Co., Md.	5/54	l, s	+[a]	0	0	0	0
4595	*Silene cucubalus*	Carroll Co., Md.	7/54	l, s, fl	+[a]	0	0	0	..
4517	*Silene noctiflora*	Haywood Co., N. C.	7/54	l, s, fl	−	0	0	0	..
4691	*Spergularia rubra*	Dorchester Co., Md.	7/54	l, s	+[a]	0	0	0	..
			CELASTRACEAE						
4617	*Euonymus Americanus*	Pulaski Co., Ky.	7/54	l, s, fl	−	0	0	0	..
			CHENOPODIACEAE						
4900	*Atriplex arenaria*	Franklin Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
4791	*Atriplex canescens*	Aguanga, Calif.	7/54	l	−	0	+	0	..
4690	*Atriplex patula* var. *hastata*	Dorchester Co., Md.	7/54	l, s, r	+[a]	0	0	0	..
4748	*Atriplex patula* var. *hastata*	Carteret Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4789	*Chenopodium album*	Aguanga, Calif.	7/54	l	+[a]	0	0	0	..
4070	*Chenopodium ambrosioides*	McIntosh Co., Ga.	4/54	l	−	0	0	0	0
4498	*Cycloloma atriplicifolia*	Lancaster Co., S. C.	7/54	l, s, r	+[a]	0	0	0	..
4771	*Suaeda linearis*	Brunswick Co., N. C.	7/54	l, s, fl, r	−	0	+	0	..
			CISTACEAE						
4280	*Hudsonia ericoides*	Pine Barrens, N. J.	6/54	l	−	0	0	+	..
4935	*Hudsonia tomentosa*	Sussex Co., Del.	8/54	l, r	−	0	0	+	..
4011	*Lechea minor*	Hammonton, N. J.	8/54	l, s, fl, r	−	0	0	+++	..
4902	*Lechea patula*	Franklin Co., Fla.	8/54	l, s, fl, r	−	0	0	+++	..
4180	*Lechea villosa*	Southport, Brunswick Co., N. C.	6/54	l, s, r	−	0	0	0	+

Accession No	Collection: Species	Collection: Source	Collection: Date Collected	Collection: Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			CLETHRACEAE						
4504	*Clethra acuminata*	Buncombe Co, N C	7/54	l, s, fl	−	0	0	++	
4193	*Clethra alnifolia*	Sampson Co, N C	6/54	l, s, fl	−	0	0	0	++
4992	*Clethra tomentosa*	Liberty Co, Fla	8/54	l, s	−	0	0	+++	
			COMMELINACEAE						
4759	*Commelina diffusa*	Carteret Co, N C	7/54	l, s, fl	−	0	0	0	
4950	*Commelina Virginica*	Leon Co, Fla	8/54	l, s, r	−	0	0	0	
4057	*Tradescantia (canaliculata) ohiensis*	McIntosh Co, Ga	4/54	l	−	0	0	0	++
			COMPOSITAE						
4593	*Acanthospermum australe*	Brunswick Co, N C	7/54	l, s	−	0	+	0	
4043	*Achillea millefolium*	Wake Co, N C	4/54	l, s	−	0	0	0	0
4543	*Actinomeris alternifolia*	Henderson Co, N C	7/54	l, s	+[a]	0	+	0	
4317	*Ambrosia artemisiifolia* var *paniculata*	Chatham Co, N C	6/54	l, s	−	0	0	0	
4277	*Ambrosia trifida*	Baltimore Co, Md	6/54	l, s	+[a]	0	0	0	
4281	*Ambrosia trifida*	Durham Co, N C	6/54	l, s, fl	+[a]	0	0	0	
4839	*Anaphalis margaritacea*	Scranton, Pa	8/54	l, s, fl, r	−	0	0	0	
4130	*Antennaria plantaginifolia*	Washington Co, Md	5/54	w	−	0	0	0	0
4301	*Anthemis arvensis*	Anne Arundel Co, Md	6/54	l, s, fl, r	−	0	0	0	
4279	*Arctium lappa*	Baltimore Co, Md	6/54	l, s	+[a]	0	0	0	
4539	*Arctium minus*	Henderson Co, N C	7/54	l, s, fl	−	0	+	0	
4629	*Artemisia absinthium*	Union Co, Ky	7/54	l, s	−	0	0	0	
4527	*Artemisia ludoviciana*	Buncombe Co, N C	7/54	l, s, r	−	0	+	0	
4793	*Artemisia tridentata* var *angustifolia*	Dripping Spring, Calif	7/54	l	−	0	+	0	
4540	*Artemisia vulgaris*	Henderson Co, N C	7/54	l, s, r	−	0	0	0	
4002	*Aspilia sp*	Tingo Maria, Peru	4/54	l, s	+[a]	+	0	0	+
4307	*Aster lateriflorus*	Chatham Co, N C	6/54	l, s, r	−	0	0	0	
4480	*Aster pilosus* var *demotus*	Elizabeth City Co, Va	7/54	l, s, fl, r	+[a]	0	0	0	
4535	*Aster puniceus*	Buncombe Co, N C	7/54	l, s	−	0	0	0	
4364	*Aster reticulatus*	Irwin Co, Ga	6/54	l, s, fl	−	0	0	0	
4974	*Balduina uniflora*	Wakulla Co, Fla	8/54	l, s, fl, r	−	+++	0	0	
4962	*Berlandiera pumila*	Leon Co, Fla	8/54	l, s, r	−	0	0	0	
4486	*Bidens bipinnata*	Nansemond Co, Va	7/54	l, s, r	−	0	0	0	
4013	*Bidens monticola*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	+++
4036	*Bidens pilosa* var *radiata*	Tingo Maria, Peru	4/54	l, s, r	−	0	0	0	++
4873	*Bidens pilosa*	Leon Co, Fla	8/54	l, s, r	−	0	+	0	
4990	*Bigelowia nudata*	Liberty Co, Fla	8/54	l, s, fl, r	−	+++	+	0	
4340	*Boltonia diffusa*	Thomas Co, Ga	6/54	l, s, r	+[a]	0	0	0	
4263	*Borrichia frutescens*	Brunswick Co, N C	6/54	l, s, fl	+[a]	0	0	0	
4244	*Cacalia atriplicifolia*	Johnston Co, N C	6/54	l, s	−	0	0	0	0
4981	*Cacalia elliottii*	Liberty Co, Fla	8/54	l, s, fl, r	−	0	0	0	
4558	*Cacalia suaveolens*	Transylvania Co, N C	7/54	l, s	−	0	0	0	
4528	*Carduus acanthoides*	Buncombe Co, N C	7/54	l, s, fl	−	0	+	0	
4422	*Carphephorus tomentosus*	Brunswick Co, N C	7/54	l, s, fl, r	−	0	0	0	
4520	*Centaurea maculosa*	Haywood Co, N C	7/54	l, s, fl, r	−	0	0	0	
4597	*Centaurea maculosa*	Allegany Co, Md	7/54	l, s, fl, r	−	0	+	0	

4643	*Centaurea solstitialis*	Pendleton Co., Ky.	7/54	l, s, fl	—	0	0	0	..
4827	*Centaurea vochinensis*	Yates Co., N. Y.	8/54	l, s, fl, r	—	+++	0	0	..
4419	*Chrysopsis gossypina*	Brunswick Co., N. C.	7/54	l, s, r	—	0	0	0	..
4420	*Chrysopsis graminifolia*	Brunswick Co., N. C.	7/54	l, s, fl, r	+[a]	0	0	0	..
5000	*Chrysopsis microcephala*	Liberty Co., Fla.	8/54	l, s, r	—	0	0	0	..
4374	*Chrysopsis pinifolia*	Moore Co., N. C.	6/54	l, s, r	+[a]	0	0	0	..
4570	*Chrysopsis trichophylla*	Brunswick Co., N. C.	7/54	l, s, r	—	0	0	0	..
4538	*Cirsium altissimum*	Henderson Co., N. C.	7/54	l, s	—	0	0	0	..
4809	*Cirsium arvense*	Yates Co., N. Y.	8/54	l, s, fl	—	0	+	0	..
4376	*Cirsium repandum*	Scotland Co., N. C.	6/54	l, s, fl, r	—	0	0	0	..
4483	*Cirsium vulgare*	Elizabeth City Co., Va.	7/54	l, s, fl	—	0	0	0	..
4039	*Clibadium psilogynum*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	+++
4982	*Coreopsis helianthoides*	Liberty Co., Fla.	8/54	l, s, r	—	0	0	0	..
4076	*Coreopsis lanceolate*	Bryan Co., Ga.	4/54	l	—	0	0	0	0
4323	*Coreopsis major* var. *stellata*	Orange Co., N. C.	6/54	l, s, fl	—	0	0	0	..
4648	*Coreopsis tripteris*	Rowan Co., Ky.	7/54	l, s, r	—	0	0	0	..
4250	*Coreopsis verticillata*	Wake Co., N. C.	6/54	l, s	—	0	0	0	0
4636	*Echinacea pollida*	Bullitt Co., Ky.	7/54	l, s, fl	+[a]	0	0	0	..
4647	*Echinacea purpurea*	Rowan Co., Ky.	7/54	l, s, fl, r	—	0	0	0	..
4702	*Eclipta alba*	Somerset Co., Md.	7/54	l, s, r	—	0	0	0	..
4425	*Elephantopus Carolinianus*	Brunswick Co., N. C.	7/54	l, s, r	—	0	+	0	..
4876	*Elephantopus elatus*	Leon Co., Fla.	8/54	l, s, fl, r	—	0	0	0	..
4468	*Erechtites hieracifolia*	Brunswick Co., N. C.	7/54	l, s, r	—	0	+	0	..
4243	*Erigeron Canadensis*	Johnston Co., N. C.	6/54	l, s, r	—	0	0	0	0
4766	*Erigeron pusillus*	Carteret Co., N. C.	7/54	l, s, r	—	0	0	0	..
4059	*Erigeron quercifolius*	McIntosh Co., Ga.	4/54	l	—	0	0	0	+
4960	*Eupatorium album*	Leon Co., Fla.	8/54	l, s, fl, r	—	0	0	0	..
4266	*Eupatorium capillifolium*	Brunswick Co., N. C.	6/54	l, s, r	—	0	0	0	..
4361	*Eupatorium compositifolium*	Emanuel Co., Ga.	6/54	l, s, r	—	0	0	0	..
4332	*Eupatorium cordigerum*	Wake Co., N. C.	6/54	l, s, fl, r	—	0	0	0	..
4770	*Eupatorium cuneifolium*	Brunswick Co., N. C.	7/54	l, s, fl, r	—	+++	0	0	..
4214	*Eupatorium fistulosum*	Wake Co., N. C.	6/54	l, s	—	0	0	0	0
4697	*Eupatorium hyssopifolium* (typical)	Somerset Co., Md.	7/54	l, s, fl, r	—	0	0	0	..
4698	*Eupatorium hyssopifolium* var. *laciniatum*	Somerset Co., Md.	7/54	l, s, fl, r	—	0	0	0	..
4554	*Eupatorium maculatum*	Macon Co., N. C.	7/54	l, s, r	—	0	0	0	..
4968	*Eupatorium mikanioides*	Wakulla Co., Fla.	8/54	l, s, fl, r	—	0	0	0	..
4437	*Eupatorium mohrii*	Brunswick Co., N. C.	7/54	l, s, r	—	+	+	0	..
4752	*Eupatorium pilosum*	Carteret Co., N. C.	7/54	l, s, fl, r	—	0	0	0	..
4937	*Eupatorium resinosum*	Sussex Co., Del.	8/54	l, s, fl, r	—	0	+	+	..
4258	*Eupatorium rotundifolium*	Johnston Co., N. C.	6/54	l, s	—	0	++	0	..
4503	*Eupatorium rugosum* var. *roanense*	Haywood Co., N. C.	7/54	l, s, r	—	0	0	0	..
4877	*Eupatorium semiserratum*	Leon Co., Fla.	8/54	l, s, fl, r	—	+++	0	0	..
4246	*Eupatorium serotinum*	Wake Co., N. C.	6/54	l, s	—	0	0	0	0
4794	*Franseria ocanthicarpa*	Dripping Spring, Calif.	8/54	l	+[a]	0	0	0	..
4966	*Gaillardia lanceolata*	Leon Co., Fla.	8/54	l, s, fl, r	—	0	0	0	..
4282	*Galinsoga parviflora*	Baltimore Co., Md.	6/54	l, s, fl, r	+[a]	0	0	0	..
4450	*Gnaphalium obtusifolium*	Brunswick Co., N. C.	7/54	l, s, fl, r	—	0	0	0	..
4575	*Gnaphalium sp.*	Brunswick Co., N. C.	7/54	l, s, fl, r	—	0	0	0	..
4803	*Gutierrezia Californica*	Murrieta, Calif.	8/54	l, s	—	0	0	0	..

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			COMPOSITAE						
4775	*Haplopappus divaricatus*	Brunswick Co., N. C.	7/54	l, s, r	+[a]	0	0	0	..
4495	*Helenium godfreyi*	Craven Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4329	*Helenium tenuifolium*	Wake Co., N. C.	6/54	l, s, fl, r	−	+	+	0	..
4331	*Helianthus microcephalus*	Orange Co., N. C.	6/54	l, s, r	−	0	0	0	..
4632	*Helianthus mollis*	Grayson Co., Ky.	7/54	l, s, r	−	0	0	0	..
4320	*Helianthus strumosus*	Chatham Co., N. C.	6/54	l, s, r	−	0	0	0	..
4471	*Helianthus tomentosus*	Brunswick Co., N. C.	7/54	l, s, r	−	0	0	0	..
4545	*Helianthus tuberosus*	Henderson Co., N. C.	7/54	l, s, r	−	0	0	0	..
4819	*Helianthus sp.*	Yates Co., N. Y.	8/54	l, s, fl, r	−	0	0	0	..
4518	*Heliopsis helianthoides*	Haywood Co., N. C.	7/54	l, s, fl, r	−	0	0	0	..
4565	*Heterotheca latifolia*	Spartanburg Co., S. C.	7/54	l, s, r	−	0	0	0	..
4131	*Hieracium venosum*	Washington Co., Md.	5/54	l, s	−	0	0	0	0
4392	*Hypochaeris radicata*	Hanover Co., Va.	7/54	l, s, fl, r	−	0	0	0	..
4665	*Inula helenium*	Allegany Co., Md.	7/54	l, s	−	0	0	0	..
4971	*Iva asperifolia*	Wakulla Co., Fla.	8/54	l, s, r	−	0	0	0	..
4472	*Iva imbricata*	Brunswick Co., N. C.	7/54	l, s	−	0	0	0	..
4234	*Lactuca Canadensis*	Durham Co., N. C.	6/54	l, s, fl, r	−	0	0	0	0
4278	*Lactuca scariola*	Baltimore Co., Md.	6/54	l, s	+[a]	0	0	0	..
4027	*Liabum pallatangense*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	..
4891	*Liatris chapmanii*	Leon Co., Fla.	8/54	l, s, r	−	0	0	0	..
4421	*Liatris graminifolia*	Brunswick Co., N. C.	7/54	l, s, r	−	0	0	0	+
4890	*Liatris laevigata*	Leon Co., Fla.	8/54	l, s, r	−	0	+	0	..
4737	*Liatris secunda*	New Hanover Co., N. C.	7/54	l, s, r	−	0	0	0	..
4627	*Liatris squarrosa*	Hopkins Co., Ky.	7/54	l, s, fl, r	−	0	0	0	..
4650	*Liatris spicata*	Lawrence Co., Ohio	7/54	l, s, fl, r	−	0	0	0	..
4967	*Melanthera hastata*	Wakulla Co., Fla.	8/54	l, s, fl	−	0	0	0	..
4590	*Mikania scandens*	Brunswick Co., N. C.	7/54	l, s	−	+	0	0	..
4875	*Parthenium hysterophorus*	Leon Co., Fla.	8/54	l, s, fl, r	−	0	+	0	..
4170	*Parthenium integrifolium*	Wake Co., N. C.	6/54	l, s, fl, r	−	0	0	0	0
4948	*Pluchea camphorata*	Leon Co., Fla.	8/54	l, s, fl, r	−	0	0	0	..
4936	*Pluchea foetida*	Sussex Co., Del.	8/54	l, s, fl, r	−	0	0	+++	..
4949	*Pluchea foetida*	Leon Co., Fla.	8/54	l, s, fl, r	−	0	0	+	..
4970	*Pluchea longifolia*	Wakulla Co., Fla.	8/54	l, s, fl, r	−	0	0	++	..
4757	*Pluchea purpurascens*	Carteret Co., N. C.	7/54	l, s	−	0	0	0	..
4344	*Pluchea rosea*	Leon Co., Fla.	6/54	l, s, fl, r	−	0	0	0	..
4790	*Pluchea sericea*	Aguanga, Calif.	7/54	l	−	0	0	0	..
4218	*Polymnia uvedalia*	Wake Co., N. C.	6/54	l, s, r	−	0	++	0	0
4178	*Pterocaulon undulatum*	Brunswick Co., N. C.	6/54	l, s	−	0	0	0	+
4616	*Ratibida pinnata*	Pulaski Co., Ky.	7/54	l, s, fl, r	−	0	0	0	..
4516	*Rudbeckia laciniata*	Haywood Co., N. C.	7/54	l, s, r	−	0	0	0	..
4189	*Rudbeckia serotina*	Brunswick Co., N. C.	6/54	l, s, fl, r	−	0	0	0	0
4991	*Senecio aureus*	Liberty Co., Fla.	8/54	l, s, r	−	0	++	0	..
4074	*Senecio Tomentosus*	Liberty Co., Ga.	4/54	l	−	0	0	0	++
4215	*Sericocarpus asterioides*	Wake Co., N. C.	6/54	l, s, fl, r	+[a]	0	0	0	0
4418	*Sericocarpus tortifolius*	Brunswick Co., N. C.	7/54	l, s, fl, r	−	+	0	0	..
4321	*Silphium asteriscus*	Orange Co., N. C.	6/54	l, s, fl	+[a]	0	0	0	..

4257	*Silphium compositum*	Wake Co, N C	6/54	l, s	−	0	0	0	
1637	*Silphium terebinthinaceum*	Bullitt Co, Ky	7/54	l, s, fl	−	0	0	0	
1291	*Silphium trifoliatum*	Quantico, Va	6/54	l, s, fl	+[a]	0	0	0	
4322	*Solidago altissima*	Chatham Co, N C	6/54	l, s, fl	+[a]	0	0	0	
4764	*Solidago boottii* var *Caroliniana*	Carteret Co, N C	7/54	l, s, fl, r	−	0	0	0	
4161	*Solidato fistulosa*	Brunswick Co, N C	7/54	l, s, r	−	0	0	0	
4553	*Solidago gigantea*	Macon Co, N C	7/54	l, s, fl	−	0	0	0	0
1185	*Solidago graminifolia*	Brunswick Co, N C	6/54	l, s, r	−	0	0	0	
4117	*Solidago minor*	Brunswick Co, N C	7/54	l, s, r	−	0	0	0	
1577	*Solidago odora*	Brunswick Co, N C	7/54	l, s, fl, r	−	0	0	0	
4261	*Solidago pinetorum*	Johnston Co, N C	6/54	l, s, r	+[a]	++	0	0	
4646	*Solidago rigida*	Rowan Co, Ky	7/54	l, s, r	−	0	0	0	
1181	*Solidago sempervirens* var *Mexicana*	Elizabeth City Co, Va	7/54	l, s, r	−	0	0	0	
4969	*Solidago stricta*	Wakulla Co, Fla	8/54	l, s, fl, r	−	0	0	0	
4946	*Solidago tortifolia*	Leon Co, Fla	8/54	l, s, r	−	0	0	0	
1825	*Sonchus oleraceus*	Yates Co, N Y	8/54	l, s, fl, r	−	0	0	0	+
4029	*Spilanthes Americana*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	
4816	*Tanacetum vulgare*	Yates Co, N Y	8/54	l, s, fl, r	−	0	0	0	
4796	*Tetradymia comosa*	Dripping Springs, Calif	7/54	l	−	0	0	0	
1692	*Tragopogon pratensis*	Accomac, Va	7/54	l, s, fl, r	−	0	0	0	
4496	*Trilisa odoratissima*	Pender Co, N C	7/54	l, s, r	−	0	0	0	
4436	*Trilisa paniculata*	Brunswick Co, N C	7/54	l, s, r	−	0	0	0	
4345	**Verbesina aristata*	Leon Co, Fla	6/54	l, s, fl, r	+[a]	0	0	0	++
1091	*Verbesina occidentalis*	Clark Co, Ga	4/54	l	−	0	0	0	
4375	*Vernonia angustifolia*	Scotland Co, N C	6/54	l, s, fl, r	−	0	0	0	
4492	*Xanthium sp*	Bertie Co, N C	7/54	l, s, r	−	0	0	0	
		CONVOLVULACEAE							
4885	*Breweria humistrata*	Leon Co, Fla	8/54	l, s, fl	−	0	0	0	
4776	*Calonyction tuba*	Brunswick Co, N C	7/54	l, s, r	+[a]	0	0	0	
4863	*Convolvulus arvensis*	Baltimore Co, Md	8/54	l, s, fl, r	−	0	0	0	
4481	*Convolvulus sepium*	Elizabeth City Co, Va	7/54	l, s, fl	−	0	0	0	
4734	*Ipomoea hederacea*	New Hanover Co, N C	7/54	l, s, r	−	0	0	0	
4896	*Ipomoea pes caprae*	Franklin Co, Fla	8/54	l, s, fr, r	−	0	0	0	
4884	*Ipomoea quamoclit*	Leon Co, Fla	8/54	l, s, fr	−	0	0	0	
4183	*Ipomoea sagittata*	Brunswick Co, N C	6/54	l, s, fl, r	−	0	0	0	0
4871	*Jacquemontia tamnifolia*	Leon Co, Fla	8/54	l, s, fl, r	−	+	0	0	
		CORNACEAE							
1272	*Cornus amomum*	Brunswick Co, N C	6/54	l, s	−	0	0	+++	
4200	*Cornus Florida*	Wake Co, N C	6/54	l, s	−	0	+	0	+++
4817	*Cornus foemina*	Yates Co, N Y	8/54	l, s	−	0	0	++	
4818	*Cornus stolonifera*	Yates Co, N Y	8/54	l, s	−	0	0	++	
		CRASSULACEAE							
4854	*Penthorum sedoides*	Frederick Co, Md	8/54	l, s, fl, r	−	0	0	++	
1612	*Sedum ternatum*	Magoffin Co, Ky	7/54	l, s, r	−	0	0	0	
		CRUCIFERAE							
1103	*Barbarea verna*	Wake Co, N C	4/54	w	−	0	0	0	++
1801	*Berteroa incana*	Yates Co, N Y	8/54	l, s, fl, r	+[a]	0	0	0	
4060	*Lepidium Virginicum*	McIntosh Co, Ga	4/54	l	−	0	0	0	+

Accession No	Species	Collection Source	Collection Date Collected	Collection Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
	CRUCIFERAE								
4101	*Cardamine hirsuta*	Wake Co , N C	4/54	w	–	0	0	0	0
4679	*Rorippa islandica (Radicula palustris)*	Mingo Co , W Va	7/54	l, s, fl	–	0	0	0	
4826	*Sisymbrium officinale*	Yates Co , N Y	8/54	l, s, r	–	0	0	0	
	CUNONIACEAE								
1040A	*Weinmannia sp*	Tingo Maria, Peru	4/54	l, s	–	++	0	++	+
4040B	*Weinmannia sp*	Tingo Maria, Peru	4/54	b	–	+	0	+++	+
	CYPERACEAE								
4907	*Bulbostylis capillaris*	Harford Co , Md	8/54	l, fl, r	–	0	0	0	
1581	*Bulbostylis stenophyllus*	Brunswick Co , N C	7/54	l	–	0	0	0	
1684	*Carex crinita*	Dorchester Co , Md	7/54	l, r	–	0	0	0	
4463	*Carex glaucescens*	Brunswick Co , N C	7/54	l, fl, r	–	0	0	0	
4235	*Carex lucida*	Durham Co , N C	6/54	s, fl, r	–	0	0	0	0
1686	*Carex lupulina*	Dorchester Co , Md	7/54	l, fl, r	–	0	0	0	
1592	*Cladium jamaicense*	Brunswick Co , N C	7/54	l, s, fl	–	0	0	0	
1568	*Cyperus lecentei*	Brunswick Co , N C	7/54	l, s, fl, r	–	+++	0	0	
4955	*Cyperus strigosus*	Leon Co , Fla	8/54	l, fl	–	0	0	+++	
4846	*Dulichium arundinaceum*	Wayne Co , Pa	8/54	l, s	–	0	0	+	
4428	*Eleocharis tuberculosa*	Brunswick Co , N C	7/54	l, s, fl, r	–	+	0	0	
4589	*Fimbristylis castanea*	Brunswick Co , N C	7/54	l	–	0	0	0	
4930	*Fuirena pumila*	Sussex Co , Del	8/54	l, fl, r	–	0	0	+	
4462	*Fuirena spuarrosa*	Brunswick Co , N C	7/54	l, s, fl, r	–	0	0	0	
4461	*Rhynchospora fascicularis*	Brunswick Co , N C	7/54	s, fl, r	–	0	0	0	
4576	*Rhynchospora macrostachya*	Brunswick Co , N C	7/54	l, s, fl, r	–	0	0	0	
4355	*Rhynchospora schoenoides*	Tift Co , Ga	6/54	l, s, fl, r	–	0	0	0	
4311	*Scirpus atrovirens* var *Georgianus*	Chatham Co , N C	6/54	l, s, fl, r	–	0	0	0	
4429	*Scirpus rubricosus (S cyperinus* var. *eriophorum)*	Brunswick Co , N C	7/54	l, s, fl	–	0	0	0	
4591	*Scirpus robustus*	Brunswick Co , N C	7/54	l	–	0	0	0	
4699	*Scirpus rubricosus*	Somerset Co , Md	7/54	l, s, fl	–	0	0	+	
4387	*Scleria triglomerata*	King and Queen Co , Va	6/54	l, s, fl	–	+	0	0	
4441	*Scleria triglomerata*	Brunswick Co , N C	7/54	l, s, fl, r	–	0	0	0	
	CRYILLACEAE								
4993	*Cliftonia monophylla*	Liberty Co , Fla	8/54	l, s	–	0	0	+++	
4171	*Cyrilla racemiflora*	Brunswick Co , N C	6/54	l, s, fl	–	+	0	+++	++
	DIAPENSIACEAE								
4412	*Pyxidanthera barbulata*	Burlington Co , N J	7/54	l, s, r	–	0	0	0	
	DIOSCOREACEAE								
4127	*Dioscorea deltoides*	Karachi, Pakistan	4/54	t	+[b]				
4705	*Dioscorea deltoides*	Murree, Pakistan	6/54	r	+[b]				
4138	*Dioscorea quaternata*	Monongalia Co , W Va	5/54	r	+[b]				
4006	*Dioscorea stegelmanniana*	Tingo Maria, Peru	4/54	l, s	+[a]				

1706	*Dioscorea sp*	Lima, Peru	7/54	t	—				
1707	*Dioscorea sp*	Lima, Peru	7/54	t	—				
			DIPSACACEAE						
1836	*Dipsacus laciniatus*	Chemung Co, N Y	8/54	1, s, fr	—	0	0	0	
4598	*Dipsacus sylvestris*	Allegany Co, Md	7/54	1, s, fl	—	0	0	0	
			DROSERACEAE						
4579	*Drosera intermedia*	Brunswick Co, N C	7/54	1, s, fl, r	—	0	0	0	
			EBENACEAE						
1061	*Diospyros Virginiana*	McIntosh Co, Ga	4/54	1	—	0	0	0	+
1126	*Diospyros Virginiana mosieri*	C G F	5/54	1, s	—	0	0	0	0
			ELAEAGNACEAE						
1014	*Elaeagnus pungens*	Wake Co, N C	4/54	1, s	—	0	0	0	+
			EMPETRACEAE						
1903	*Ceratiola ericoides*	Franklin Co, Fla	8/54	1, s	—	0	0	+++	
			EQUISETACEAE						
1395	*Equisetum arvense*	Baltimore Co, Md	7/54	1, s, r	—	0	0	0	
4776	*Equisetum hyemale* var *affine*	Baltimore Co, Md	7/54	s, r	—	0	0	0	
			ERICACEAE						
1409	*Arctostaphylos uva-ursi*	Ocean Co, N J	7/54	1, s, r	—	+	0	++	
1410	*Chamaedaphne calyculata*	Burlington Co, N J	7/54	1, s, fl	—	++	0	+	
1397	*Epigaea repens*	Atlantic Co, N J	7/54	1, s, r	—	0	0	0	
1067	*Gaylussacia tomentosa*	McIntosh Co, Ga	4/54	1	—	0	0	++	+
1302	*Kalmia angustifolia*	Anne Arundel Co, Md	6/54	1, s, fr	—	0	0	++	
4411	*Leiophyllum buxifolium*	Burlington Co, N J	7/54	1, s, r	—	0	0	+	
4169	*Leiophyllum buxifolium* var *hugeri*	Brunswick Co, N C	6/54	1, s, fl	—	+++	0	++	+++
4194	*Lyonia ligustrina*	Sampson Co, N C	6/54	1, s	—	+++	0	0	+
1177	*Lyonia mariana*	Brunswick Co, N C	6/54	1, s, fl, r	—	+++	0	0	+
1663	*Menziesia pilosa*	Garrett Co, Md	7/54	1, s	—	0	0	0	
1191	*Oxydendrum arboreum*	Sampson Co, N C	6/54	1, s	—	+++	0	0	+
4658	*Pyrola rotundifolia* var *Americana*	Preston Co, W Va	7/54	1	—	0	0	0	
4843	*Rhododendron Canadense*	Wayne Co, Pa	8/54	1, s, r	—	0	0	++	
1105	*Rhododendron viscosum*	Atlantic Co, N J	7/54	1, s, fl	—	0	0	+	
1810	*Vaccinium corymbosum*	Wayne Co, Pa	8/54	1, s, fr	—	0	0	++	
4916	*Vaccinium macrocarpon*	Atsion, N J	8/54	1, s, fr, r	—	+++	0	+	
4066	*Vaccinium myrsinites*	McIntosh Co, Ga	4/54	1	—	0	0	++	+
1300	*Vaccinium stamineum*	Anne Arundel Co, Md	6/54	1, s, fr	—	0	0	++	
4192	*Vaccinium sp*	Sampson Co, N C	6/54	1, s	—	+	0	0	+
4196	*Zenobia pulverulenta forma nuda*	Sampson Co, N C	6/54	1, s, fr	—	+++	0	++	++
			ERIOCAULACEAE						
4989	*Eriocaulon decangulare*	Liberty Co, Fla	8/54	1, s, fr, r	—	0	0	0	
4917	*Eriocaulon decangulare*	Atsion, N J	8/54	1, s, fl, r	—	0	0	0	
			EUPHORBIACEAE						
1630	*Acalypha Virginica*	Union Co, Ky	7/54	1, s, fl, r	—	0	0	0	
4882	*Aleurites fordii*	Leon Co, Fla	8/54	1, s	—	0	0	+++	
1385A	*Aleurites Montana*	C G F	7/54	1	—	0	0	++	

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4385B	*Aleurites Montana*	C. G. F.	7/54	s	–	0	0	0	. .
4715	*Antidesma platyphyllum*	C. G. F.	7/54	l, s, fr	–	0	0	+	. .
4788	*Croton Californicus*	Aguanga, Calif.	7/54	l, s	–	0	0	+++	. .
4367	*Croton glandulosus*	Richmond Co., Ga.	6/54	l, s, fl, r	–	0	0	0	. .
4618	*Croton monanthogynus*	Pulaski Co., Ky.	7/54	l, s, r	–	0	0	0	. .
4893	*Euphorbia (Poinsettia) dentata*	Leon Co., Fla.	8/54	l, s, fl	–	0	0	0	. .
4913	*Euphorbia esula*	Hammonton, N. J.	8/54	l, s, r	–	0	0	0	. .
4398	*Euphorbia ipecacuanhae*	Atlantic Co., N. J.	7/54	l, s, r	–	0	0	0	. .
4488	*Euphorbia maculata*	Nansemond Co., Va.	7/54	l, s, r	–	0	0	0	. .
4735	*Euphorbia serpens*	New Hanover Co., N. C.	7/54	l, s, fl, r	–	0	0	0	. .
4898	*Stillingia aquatica*	Franklin Co., Fla.	8/54	l, s, fl	–	0	0	+++	. .
4186	*Stillingia sylvatica*	Brunswick Co., N. C.	6/54	l, s, fl	–	+++	0	++	0
		FAGACEAE							
4274	*Castanea ashei*	Brunswick Co., N. C.	6/54	l, s	–	0	0	+++	
4532	*Castanea dentata*	Buncombe Co., N. C.	7/54	l, s	–	0	0	+++	. .
4290	*Castanea pumila*	Anne Arundel Co., Md.	6/54	l, s, fl	–	0	0	+++	. .
4201	*Fagus grandifolia*	Wake Co., N. C.	6/54	l, s	–	0	+	0	+++
4308	*Quercus alba*	Chatham Co., N. C.	6/54	l, s	–	0	0	++	. .
4929	*Quercus falcata*	Sussex Co., Del.	8/54	l, s, fr	–	0	0	++	. .
4285	*Quercus ilicifolia*	Anne Arundel Co., Md.	6/54	l, s, fr	–	0	0	+++	. .
4586	*Quercus marilandica*	Brunswick Co., N. C.	7/54	l, s	–	0	0	++	. .
4921	*Quercus phellos*	Charles Co., Md.	8/54	l, s	–	0	0	+++	. .
4401	*Quercus prinoides*	Atlantic Co., N. J.	7/54	l, s	–	0	0	0	. .
4533	*Quercus prinus*	Buncombe Co., N. C.	7/54	l, s	–	0	0	++	. .
4075	*Quercus pumila*	Liberty Co., Ga.	4/54	l	–	0	0	++	. .
4736	*Quercus stellata* var. *margaretta*	New Hanover Co., N. C.	7/54	l, s	–	0	0	0	+
4435	*Quercus Virginiana*	Brunswick Co., N. C.	7/54	l, s	–	0	0	+++	. .
		GENTIANACEAE							
4466	*Nymphoides aquatica*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	
4908	*Nymphoides peltata*	Elmer, N. J.	8/54	l, s, fr, r	+[a]	0	0	0	. .
4850	*Sabatia angularis*	Baltimore Co., Md.	8/54	l, s, fl, r	–	0	0	0	. .
4491	*Sabatia difformis*	Onslow Co., N. C.	7/54	l, s, fl, r	–	0	0	0	. .
4499	*Sabatia kennedyana*	Horry Co., S. C.	7/54	l, s, fl	–	0	0	0	. .
4624	*Swertia Caroliniensis*	Clinton Co., Ky.	7/54	l, s, fl, r	–	0	0	0	. .
		GERANIACEAE							
4137	*Geranium maculatum*	Monongalia Co., W. Va.	5/54	l, s, fl, r	–	0	0	+	+
		GRAMINEAE							
4939	*Ammophila breviligulata*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	0	
4415	*Amphicarpum purshii*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	. .
4578	*Andropogon scoparius*	Brunswick Co., N. C.	7/54	l, s, r	–	++	0	0	. .
4751	*Andropogon Virginicus* var. *glaucopsis*	Carteret Co., N. C.	7/54	l	–	+++	0	+	. .
4910	*Aristida oligantha*	Hammonton, N. J.	8/54	l, r	–	0	+	0	. .
4742	*Aristida stricta*	New Hanover Co., N. C.	7/54	l, fl, r	–	0	0	+	. .
4271	*Arundinaria tecta*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	. .
4446	*Arundo donax*	Brunswick Co., N. C.	7/54	l, s	–	0	+++	0	. .

4721	*Axonopus compressus*	New Hanover Co., N. C.	7/54	l, fr, r	–	0	0	0	..
4696	*Cenchrus tribuloides*	Worchester Co., Md.	7/54	l, r	–	0	0	0	..
4753	*Cenchrus tribuloides*	Carteret Co., N. C.	7/54	l, fl, r	–	0	0	0	..
4432	*Ctenium aromaticum*	Brunswick Co., N. C.	7/54	l, s, fr, r	–	+	0	0	..
4722	*Cynodon dactylon*	New Hanover Co., N. C.	7/54	l, s, r	–	0	0	0	..
4567	*Digitaria sanguinalis*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4585	*Echinochloa walteri*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4487	*Eleusine indica*	Nansemond Co., Va.	7/54	l, s, fl, r	–	0	0	0	..
4240	*Elymus Virginicus*	Johnston Co., N. C.	6/54	s, r	–	0	0	0	0
4681	*Eragrostis megastachya*	Dorchester Co., Md.	7/54	l, s, r	–	0	0	0	..
4906	*Eragrostis pilosa*	Anne Arundel Co., Md.	8/54	l, r	–	0	0	0	..
4938	*Eragrostis spectabilis*	Sussex Co., Del.	8/54	l, fl, r	–	0	0	0	..
4219	*Hystrix patula*	Wake Co., N. C.	6/54	l, s, r	–	0	0	0	0
4447	*Leersia oryzoides*	Brunswick Co., N. C.	7/54	l	–	0	0	0	..
4768	*Panicum amarum*	Brunswick Co., N. C.	7/54	l, r	+[a]	0	0	0	..
4451	*Panicum hemitomon*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4326	*Panicum scoparium*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4687	*Panicum scoparium*	Dorchester Co., Md.	7/54	l, s, fl, r	–	0	0	0	..
4268	*Panicum virgatum*	Brunswick Co., N. C.	6/54	l, fl, r	+[a]	0	0	0	..
4256	*Paspalum dilatatum*	Wake Co., N. C.	6/54	s, r	–	0	0	0	0
4746	*Paspalum Floridanum* var. *glabratum*	Carteret Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
4168	*Paspalum urvillei*	Brunswick Co., N. C.	6/54	l, s, fl	–	+	0	0	+
4733	*Phragmites communis* var. *berlandieri*	New Hanover Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4694	*Polypogon monspeliensis*	Somerset Co., Md.	7/54	s, fl, r	–	0	0	0	..
4931	*Sacciolepis striata*	Sussex Co., Del.	8/54	l, fr, r	–	0	0	0	..
4584	*Setaria magna*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4315	*Sorghum halapense*	Wake Co., N. C.	6/54	l, s, fr	–	0	0	0	..
4445	*Spartina cynosuroides*	Brunswick Co., N. C.	7/54	l, s, fl	–	0	0	0	..
4408	*Spartina patens*	Parkertown, Atlantic Co., N. J.	7/54	l, s, r	–	0	0	0	..
4762	*Spartina patens*	Carteret Co., N. C.	7/54	l, fl, r	–	0	0	0	..
4980	*Stenotaphrum secundatum*	Wakulla Co., Fla.	8/54	l, fl, r	–	0	0	0	..
4924	*Triodia flava*	Charles Co., Md.	8/54	l, fl, r	–	0	0	0	..
4942	*Triplasis purpurea*	Sussex Co., Del.	8/54	l, s, fl, r	–	0	0	+++	..
4255	*Tripsacum dactyloides*	Wake Co., N. C.	6/54	l, s, fl, r	–	0	0	0	0
4306	*Uniola latifolia*	Chatham Co., N. C.	6/54	l, s, fl, r	–	0	0	0	..
4920	*Uniola laxa*	Charles Co., Md.	8/54	l, r	–	0	0	0	..
4474	*Uniola paniculata*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	0	0	..
4444	*Zizania aquatica*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	..
4723	*Zizaniopsis miliacea*	New Hanover Co., N. C.	7/54	l, s	–	0	0	0	..
		GUTTIFERAE							
4430	*Ascyrum hyperidoides*	Brunswick Co., N. C.	7/54	l, s, r,	–	0	0	0	..
4342	*Hypericum densiflorum*	Leon Co., Fla.	6/54	l, s, fl	–	0	0	+	..
4559	*Hypericum densiflorum*	Macon Co., N. C.	7/54	l, s, fl	–	+	0	+	..
4633	*Hypericum dolabriforme*	Bullitt Co., Ky.	7/54	l, s, fl	–	+	0	0	..
4172	*Hypericum galioides*	Brunswick Co., N. C.	6/54	l, s, fl, r	–	+++	0	0	0
4297	*Hypericum (Sarothra) gentianoides*	Caroline Co., Va.	6/54	l, r	–	0	0	0	..
4343	*Hypericum myrtifolium*	Leon Co., Fla.	6/54	l, s, fl	–	0	0	++	..
4572	*Hypericum nudiflorum*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	+++	0	+++	..
4389	*Hypericum perforatum*	Hanover Co., Va.	7/54	l, s, fl, r	–	0	0	0	..

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4814	*Hypericum punctatum*	Wayne Co, Pa	8/54	l, s, fl	–	0	0	++	
4613	*Hypericum spathulatum*	Clay Co, Ky	7/54	l, s, fl	–	++	0	+	
4156	*Hypericum tubulosum* var *walteri*	Brunswick Co, N C	7/54	l, s, fl	–	0	0	+	
4915	*Hypericum Virginicum*	Atsion, N J	8/54	l, s, fl, r	–	0	0	++	
		HAEMODORACEAE							
4116	*Lachnanthes tinctoria*	Brunswick Co, N C	7/54	l, s, fl, r	–				
4918	*Lachnanthes tinctoria*	Hammonton, N J	8/54	l, s, fl, r	–	0	0	0	
		HALORAGACEAE							
4933	*Proserpinaca palustris*	Sussex Co, Del	8/54	l, s, r	–	0	0	+++	
		HAMAMELIDACEAE							
4424	*Hamamelis Virginiana*	Brunswick Co, N C	7/54	l, s	–	0	0	+	
		HIPPOCASTANACEAE							
4147	*Aesculus octandra*	Preston Co, W Va	5/54	l, s	–	++	0	0	+
4505	*Aesculus octandra*	Haywood Co, N C	7/54	l, s	–	+	0	0	
		HYDROCHARITACEAE							
4926	*Vallisneria Americana*	Charles Co, Md	8/54	l, r	–	0	0	0	
4978	*Vallisneria Americana*	Wakulla Co, Fla	8/54	l, s, r	–	0	0	0	
		HYDROPHYLLACEAE							
4675	*Hydrophyllum Canadense*	Harford Co, Md	7/54	l, s, r	–	0	0	0	
4975	*Nama corymbosum*	Wakulla Co, Fla	8/54	l, s, fl, r	–	0	0	0	
		IRIDACEAE							
4634	*Belamcanda Chinensis*	Bullitt Co, Ky	7/54	l, s, r	–	0	0	0	
		ISOETACEAE							
4864	*Isoetes riparia*	Harford Co, Md	8/54	l, s	–	0	0	0	
		JUGLANDACEAE							
4739	*Carya cordiformis*	New Hanover Co, N C	7/54	l, s	–	++	0	++	
4198	*Carya tomentosa*	Wake Co, N C	6/54	l	–	+++	0	0	0
4209	*Juglans nigra*	Wake Co, N C	6/54	l, s	–	++	0	++	0
		JUNCACEAE							
4932	*Juncus acuminatus*	Sussex Co, Del	8/54	l, fl, r	–	0	0	0	
4187	*Juncus biflorus*	Brunswick Co, N C	6/54	s, fl, r	–	++	0	0	0
4127	*Juncus Canadensis*	Brunswick Co, N C	7/54	l, s, fl, r	–	0	0	0	
4940	*Juncus Canadensis*	Sussex Co, Del	8/54	l, fl, r	–	0	0	0	
4126	*Juncus coriaceus*	Brunswick Co, N C	7/54	s, fl	–	0	0	0	
4582	*Juncus repens*	Brunswick Co, N C	7/54	l, s, fl, r	–	+++	0	0	
4919	*Juncus scirpoides*	Charles Co, Md	8/54	l, fl	–	0	0	0	
		LABIATAE							
4868	*Agastache nepetoides*	Harford Co, Md	8/54	l, s, fl, r	–	0	0	0	
4813	*Blephilia hirsuta*	Yates Co, N Y	8/54	l, s, fl, r	+[a]	0	0	0	
4557	*Collinsonia Canadensis*	Macon Co, N C	7/54	l, s, r	–	0	0	0	
4999	*Conradina canescens*	Liberty Co, Fla	8/54	l, s, r	–	+	0	0	

4625	*Cunila origanoides*	Hopkins Co , Ky	7/54	l, s, r	—	0	0	0	
1778	*Glechoma hederacea*	Baltimore Co , Md	7/54	l, s, r	—	0	0	0	
4296	*Hedeoma pulegioides*	Charles Co , Md	6/54	l, s, r	—	0	0	0	
1871	*Hyptis mutabilis*	Leon Co , Fla	8/54	l, s, r	—	0	0	0	
1951	*Hyptis radiata*	Leon Co , Fla	8/54	l, s, r	—	0	0	0	
4878	*Leonotis nepetaefolia*	Leon Co , Fla	8/54	l, s, fl	—	0	0	0	
1807	*Leonurus cardiaca*	Yates Co , N Y	8/54	l, s, fl	+[a]	0	0	0	
1822	*Lycopus Americanus*	Yates Co , N Y	8/54	l, s, r	—	0	0	0	
1541	*Mentha piperita*	Henderson Co , N C	7/54	l, s, r	—	0	0	0	
1596	*Mentha piperita*	Washington Co , Md	7/54	l, s, fl, r	—	0	0	0	
1319	*Monarda fistulosa*	Orange Co , N C	6/54	l, s, fl	—	0	0	0	
4905	*Monarda punctata*	Anne Arundel Co , Md	8/54	l, s, fl, r	—	0	0	0	
1891	*Monarad punctata*	Franklin Co , Fla	8/54	l, s, fl	—	0	0	0	
1521	*Nepeta cataria*	Buncombe Co , N C	7/54	l, s, fl, r	—	0	0	0	
4599	*Nepeta cataria*	Allegany Co , Md	7/54	l, s, fl	—	0	0	0	
1782	*Perilla frutescens*	Harford Co , Md	7/54	l, s, r	—	0	0	0	
4609	*Pycanthemum clinopodioides*	Wyoming Co , W Va	7/54	l, s, fl, r	—	0	0	0	
1239	*Pycanthemum flexuosum*	Durham Co , N C	6/54	l, s, fl	—	+	0	0	0
1293	*Pycnanthemum tenuifolium*	Charles Co , Md	6/54	l, s, fl, r	—	+	0	0	
1619	*Physostegia Virginiana*	Rowan Co , Ky	7/54	l, s, fl, r	—	0	0	0	
1362	*Salvia coccinea*	Toombs Co , N C	6/54	l, s, fl, r	+[a]	0	0	0	
4802	*Salvia mellifera*	Murrieta, Calif	7/54	w	—	+	0	0	
4051	*Scutellaria incana*	Gilmer Co , W Va	7/54	l, s, fl, r	—	0	0	0	
1273	*Scutellaria integrifolia*	Brunswick Co , N C	6/54	l, s, fl	—	++	0	0	
1316	*Stachys italica*	Thomas Co , Ga	6/54	l, s, r	—	0	0	0	
1476	*Teucrium Canadense*	Elizabeth City Co , Va	7/54	l, s, fl, r	—	0	0	0	
1952	*Trichostema dichotomum*	Leon Co , Fla	8/54	l, s, r	—	0	0	0	
		LARDIZABALACEAE							
1521	*Akebia quinata*	Henderson Co , N C	7/54	l, s, fl	—	0	0	0	
1530	*Akebia quinata*	Buncombe Co , N C	7/54	l, s	—	0	0	0	
		LAURACEAE							
1190	*Sassafras albidum*	Sampson Co , N C	6/54	l, s	—	++	0	0	0
		LEGUMINOSAE							
4124	*Acacia caffra*	C G F	5/54	l, s	—	0	0	0	++
1158	*Acacia chloriophylla*	C G F	5/54	l, s	—	++	0	0	++
1160	*Acacia concinna*	C G F	5/54	l, s	+[a]	0	0	0	+
1159	*Acacia detinens*	C G F	5/54	l, s	—	0	0	0	0
1161	*Acacia farnesiana*	C G F	5/54	l, s	—	0	+	0	+
4162	*Acacia galpinii*	C G F	5/54	l, s	+[a]	0	0	0	+
1163	*Acacia koa*	C G F	5/54	l, s, fr	—	++	0	0	++
1164	*Acacia laeta*	C G F	5/54	l, s	+[a]	0	0	0	++
4165	*Acadia pallens*	C G F	5/54	l, s	—	+++	0	0	+++
1729	*Aeschynomone indica*	New Hanover Co , N C	7/54	l, s, fl, r	—	0	0	0	
4229A	*Albizzia adinocephala*	C G F	6/54	l	+[a]	0	0	0	+++
4229B	*Albizzia adinocephala*	C G F	6/54	s	+[a]	0	0	0	0
1381	*Albizzia caribaea*	C G F	7/54	l, s	—	0	++	0	
4382	*Albizzia chevalieri*	C G F	7/54	l, s	—	0	0	0	
1225	*Albizzia Chinensis*	C G F	6/54	l, s	—	0	0	++	++
4228A	*Albizzia corbissieri*	C G F	6/54	l	+[a]	0	0	0	++

Accession No	Species	Collection Source	Date Collected	Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
		LEGUMINOSAE							
4228B	*Albizzia corbissieri*	C G F	6/54	s	+[a]	0	0	0	0
4230A	*Albizzia falcata*	C G F	6/54	l	+[a]	0	0	++	++
4230B	*Albizzia falcata*	C G F	6/54	s	+[a]	0	0	0	++
4312	*Albizzia julibrissin*	Chatham Co , N C	6/54	l, s	+[a]	0	0	0	
1231A	*Albizzia lucida*	C G F	6/54	l	+[a]	0	+	0	+
4231B	*Albizzia lucida*	C G F	6/54	s	+[a]	0	+++	0	+
4232A	*Albizzia mollis*	C G F	6/54	l	+[a]	0	0	0	0
4232B	*Albizzia mollis*	C G F	6/54	s	+[a]	0	0	0	0
4233A	*Albizzia richardiana*	C G F	6/54	l	−	0	++	0	++
1233B	*Albizzia richardiana*	C G F	6/54	s	+[a]	0	0	0	+
1226	*Albizzia sassa*	C G F	6/54	l, s	−	0	0	0	0
1227	*Albizzia zygia*	C G F	6/54	l, s	−	0	0	0	0
4175	*Amorpha cyanostachya*	Brunswick Co , N C	6/54	l, s, fl	−	++	0	0	++
4925	*Amorpha cf fruticosa*	Charles Co , Md	8/54	l, s, fr	−	0	0	+++	
4423	*Amorpha herbacea*	Brunswick Co , N C	7/54	l, s, fl	−	0	0	0	
4795	*Astragalus pomonensis*	Dripping Springs, Calif	7/54	l	−	0	0	0	
4700	*Baptisia alba*	Accomac, Va	7/54	l, s, fr, r	−	0	+++	0	
4372	*Baptisia cinerea*	Moore Co , N C	6/54	l, s	−	++	0	0	
4339	*Baptisia psammophila*	Leon Co , Fla	6/54	l, s, fr, r	−	0	++	0	
4242	*Baptisia tinctoria*	Wake Co , N C	6/54	l, s, fl, r	−	+	+	0	0
4284	*Baptisia tinctoria*	Alexandria, Va	6/54	l, s, fl	−	+	++	0	
4482	*Cassia fasciculata*	Elizabeth City Co , Va	7/54	l, s, r	−	0	0	0	
4888	*Cassia cf littoralis*	Leon Co , Fla	8/54	l, s	−	0	0	+++	
4848	*Cassia Marilandica*	Baltimore Co , Md	8/54	l, s, fl, r	−	0	0	0	
4889	*Cassia cf multipinnata*	Leon Co , Fla	8/54	l, s, r	−	0	+	0	
4569	*Cassia nictitans*	Brunswick Co , N C	7/54	l, s, r	−	+	0	0	
4879	*Cassia occidentalis*	Leon Co , Fla	8/54	l, s, fr	−	0	0	0	
4338	*Cassia tora*	Leon Co , Fla	6/54	l, s, r	−	0	0	0	
4587	*Cassia tora*	Brunswick Co , N C	7/54	l, s, fl	−	0	0	0	
4760	*Centrosema Virginianum*	Carteret Co , N C	7/54	l, s, fr	−	0	0	0	
4725	*Clitoria mariana*	New Hanover Co , N C	7/54	l, s, r	−	0	0	0	
4849	*Coronilla varia*	Baltimore Co , Md	8/54	l, s, r	−	0	0	0	
4957	*Crotalaria angulata*	Leon Co , Fla	8/54	l, s, fr, r	−	0	+	0	
4945	*Crotalaria cf mucronata*	Leon Co , Fla	8/54	l, s, fr, r	−	0	0	0	
4872	*Crotalaria spectabilis*	Leon Co , Fla	8/54	l, s, r	−	0	0	++	
4034	*Crotilaria sp*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	
4356	*Daubentonia punicea*	Irwin Co , Ga	6/54	l, s, fr	+[a]	0	0	0	++
4838	*Desmodium Canadense*	Bradford Co , Pa	8/54	l, s, fl	−	0	0	0	
4571	*Desmodium ciliare*	Brunswick Co , N C	7/54	l, s, fl, r	−	0	0	0	
4964	*Desmodium lineatum*	Leon Co , Fla	8/54	l, s, r	−	+	0	0	
4337	*Desmodium paniculatum*	Thomas Co , Ga	6/54	l, s, r	−	0	0	++	
4727	*Desmodium tenuifolium*	New Hanover Co , N C	7/54	l, s, fl, r	−	0	0	0	0
4728	*Desmodium tortuosum*	New Hanover Co , N C	7/54	l, s, r	−	0	0	0	
4965	*Desmodium viridiflorum*	Leon Co , Fla	8/54	l, s, fl, r	+[a]	0	0	0	
4977	*Galactia mollis*	Wakulla Co , Fla	8/54	l, s, fl	−	0	0	+++	
4371	*Galactia regularis*	Moore Co , N C	6/54	l, s, fl	−	0	0	+	

1880	*Gleditsia triacanthos*	Leon Co, Fla	8/54	l, s, fr	–	0	0	++	
1053	*Glottidium vesicarium*	McIntosh Co, Ga	4/54	l	–	0	0	0	++
4611	*Gymnocladus dioica*	Mason Co, Ky	7/54	l, s	–	0	0	0	
1179	*Indigofera Caroliniana*	Brunswick Co, N C	6/54	l, s	–	0	0	0	+
4500	*Lathyrus latifolius*	Spartenberg Co, S C	7/54	l, s, fl, fr	–	0	0	0	
1954	*Lespedeza angustifolia*	Leon Co, Fla	8/54	l, s, fl, r	–	0	0	+++	
4759	*Lespedeza bicolor*	Carteret Co, N C	7/54	l	–	0	0	0	
1373	*Lespedeza capitata*	Scotland Co, N C	6/54	l, s, fl	–	0	0	++	
4413	*Lespedeza capitata*	Camden Co, N J	7/54	l, s, fl	–	0	0	+	
1251	*Lespedeza cuneata*	Wake Co, N C	6/54	l, s	–	0	0	0	0
4741	*Lespedeza hirta* var *appressipilis*	New Hanover Co, N C	7/54	l, s, fl, r	–	0	0	++	
4205	*Lespedeza procumbens*	Wake Co, N C	6/54	l, s, r	–	0	0	0	+
1963	*Lespedeza repens*	Leon Co, Fla	8/54	l, s	–	0	0	+++	
4113	*Lespedeza stuevei*	Brunswick Co, N C	7/54	l, s, fl	–	0	0	+++	
4212	*Lespedeza Virginica*	Wake Co, N C	6/54	l, s, r	–	+	0	0	0
1811	*Lotus corniculatus*	Yates Co, N Y	8/54	l, s, fl	–	0	0	0	
4800	*Lupinus excubitus*	Dripping Springs, Calif	7/54	w	–	0	+++	0	
4077	*Lupinus nuttallii*	Bryan Co, Ga	4/54	l, s	–	0	+++	0	+
4851	*Medicago lupulina*	Baltimore Co, Md	8/54	l, s, fl, r	+[a]	0	0	0	
4852	*Medicago sativa*	Carroll Co, Md	8/54	l, s, fl, r	+[a]	0	0	0	
4207	*Melilotus alba*	Wake Co, N C	6/54	l, s, fl, r	–	0	0	0	0
1639	*Petalostemum candidum*	Bullitt Co, Ky	7/54	l, s, fl, r	–	0	0	0	
1901	*Petalostemum feayi*	Franklin Co, Fla	8/54	l, s, fl	–	0	0	+	
4887	*Petalostemum pinnatum*	Leon Co, Fla	8/54	l, s, fl, r	–	0	0	0	
4997	*Pitcheria galactioides*	Liberty Co, Fla	8/54	l, s, r	–	+	0	0	
4363	*Psoralea canescens*	Toombs Co, Ga	6/54	l, s, fl, r	–	0	0	0	
4238	*Psoralea psoralioides*	Durham Co, N C	6/54	l, s, fl	+[a]	0	0	0	0
4611	*Psoralea psoralioides* var *eglandulosa*	Laurel Co, Ky	7/54	l, s, fl	–	0	0	0	
4017	*Pueraria lobata*	Wake Co, N C	4/54	l, s	–	0	0	0	
4099	*Pueraria lobata*	Clarke Co, Ga	4/54	l	–	0	0	0	+++
4493	*Rhynchosia difformis*	Pender Co, N C	7/54	l, s, fl, r	–	+	0	0	
1959	*Rhynchosia simplicifolia*	Leon Co, Fla	8/54	l, s, fl, r	–	0	0	0	
4467	*Robinia cf nana*	Brunswick Co, N C	7/54	l, s	–	0	0	0	
4522	*Schrankia microphylla*	Henderson Co, N C	7/54	l, s, fl	–	0	0	0	
4726	*Strophostyles helvola*	New Hanover Co, N C	7/54	l, s, r	–	0	0	0	
4958	*Stylosanthes biflora*	Leon Co, Fla	8/54	l, s, fl, r	–	0	0	0	
4580	*Tephrosia Florida*	Brunswick Co, N C	7/54	l, s, fr, r	–	++	0	0	
4211	*Tephrosia Virginiana*	Wake Co, N C	6/54	l, s	–	++	+	0	0
4012	*Tephrosia sp*	Tingo Maria, Peru	4/54	l, s	–	+	0	0	0
4101	*Vicia villosa*	Wake Co, N C	4/54	l, s	–	0	0	0	+
1721	*Vigna luteola*	New Hanover Co, N C	7/54	l, s, fr	–	0	0	0	
4774	*Vigna sinensis*	Brunswick Co, N C	7/54	l, s, r	–	0	0	0	
1184	*Zornia bracteata*	Brunswick Co, N C	6/54	l, s, r	–	0	0	0	0
		LEMNACEAE							
1781	*Spirodela polyrhiza* and *Lemna sp*	Harford Co, Md.	7/54	l	–	0	0	0	..
		LENTIBULARIACEAE							
1909	*Utricularia sp*	Elmer, N J	8/54	l	–	0	0	0	..

Accession No	Collection: Species	Collection: Source	Collection: Date Collected	Collection: Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			LILIACEAE						
4351	*Aletris aurea*	Tift Co, Ga	6/54	l, s, fl, r	+[a]	0	0	0	
4721	*Asparagus racemosus*	C G F	7/54	l, s	+[a]	0	0	0	
4653	*Clintonia umbellulata*	Monongalia Co, W Va	7/54	l, s, r	+[b]	0	0	0	
4651	*Disporum lanuginosum*	Monongalia Co, W Va	7/54	l, s, fr	−	0	0	0	
4142	*Erythronium Americanum*	Monongalia Co, W Va	5/54	l, s	−	0	+	0	++
4563	*Lilium superbum*	Macon Co, N C	7/54	l, s, fl, r	−	0	++	0	
4855	*Medeola Virginiana*	Frederick Co, Md	8/54	l, s, fr, r	−	0	0	0	
4357	*Melanthium Virginicum*	Irwin Co, Ga	6/54	l, s, fl, r	−	0	++	0	
4018	*Polygonatum biflorum*	Wake Co, N C	4/54	l, s	−	0	0	0	0
4019	*Smilacina racemosa*	Wake Co, N C	4/54	l, s	−	0	0	0	+
4088	*Smilax rotundifolia*	Clarke Co, Ga	4/54	l, s	−	0	0	0	0
4979	*Smilax sp*	Leon Co, Fla	8/54	t	−	0	0	+	
4678	*Trillium flexipes*	Harford Co, Md	7/54	l, s, fr	+[b]	0	0	0	
4146	*Trillium grandiflorum*	Preston Co, W Va	5/54	l, s, r	+[a]	0	0	0	0
4988	*Zigadenus glaberrimus*	Liberty Co, Fla	8/54	l, s, fl, r	−	0	0	+	
			LINACEAE						
4704	*Linum Floridanum*	Onslow Co, N C	7/54	l, s, fl, r	−	0	0	+	
4388	*Linum Virginianum*	Caroline Co, Va	6/54	l, s, fl, r	−	0	0	0	
			LOBELIACEAE						
4695	*Lobelia cardinalis*	Somerset Co, Md	7/54	l, s, fl, r	−	0	++	0	
4985	*Lobelia glandulifera*	Liberty Co, Fla	8/54	l, s, r	−	0	0	0	
			LOGANIACEAE						
4454	*Polypremum procumbens*	Brunswick Co, N C	7/54	l, s, fl	−	0	0	0	
			LYCOPODIACEAE						
4761	*Lycopodium alopecuroides*	Carteret Co, N C	7/54	w	−	0	++	0	
4314	*Lycopodium complanatum* var *flabelliforme*	Chatham Co, N C	6/54	w	−	0	+++	0	
4404	*Lycopodium inundatum* var *bigelovii*	Atlantic Co, N J	7/54	w, r	−	0	+++	0	
4657	*Lycopodium lucidulum*	Preston Co, W Va	7/54	w	−	0	+++	0	
			LYTHRACEAE						
4934	*Decodon verticillatus*	Sussex Co, Del	8/54	l, s, fl	−	0	0	+++	
4449	*Lagerstroemia indica*	Brunswick Co, N C	7/54	l, s, fl	−	0	0	0	
4972	*Lythrum lineare*	Wakulla Co, Fla	8/54	l, s	−	0	0	+++	
			MAGNOLIACEAE						
4093	*Liriodendron tulipifera*	Clarke Co, Ga	4/54	l	−	0	+	0	+
4623	*Magnolia acuminata*	Wayne Co, Ky	7/54	l, s	−	0	++	0	
4511	*Magnolia fraseri*	Haywood Co, N C	7/54	l, s	−	0	0	0	
4222	*Magnolia tripetala*	Wake Co, N C	6/54	l, s, fr	−	0	0	0	0
4080	*Magnolia Virginiana*	Bulloch Co, Ga	4/54	l	−	0	0	0	0
			MALVACEAE						
4174	*Abutilon theophrasti*	Baltimore Co, Md	6/54	l, s, r	−				
4354	*Hibiscus aculeatus*	Tift Co, Ga	6/54	l, s, fr, r	−	0	0	0	
4359	*Hibiscus militaris*	Irwin Co, Ga	6/54	l, s, fl, r	−	0	0	0	

1631	*Hibiscus militaris*	Daviess Co, Ky	7/54	l, s	—	0	0	0	
1275	*Hibiscus moscheutos*	Chatham Co, N C	6/54	l, s	—	0	0	0	
1701	*Hibiscus trionum*	Somerset Co, Md[b]	7/54	l, s, fl, r	—	0	0	0	
1177	*Kosteletzkya Virginica*	Elizabeth City Co, Va	7/54	l, s, r	—	0	0	0	
1600	*Malva moschata*	Garrett Co, Md	7/54	l, s, fl, r	+[a]	0	0	0	
1805	*Malva rotundifolia*	Yates Co, N Y	8/54	l, s, fl, r	—	0	0	0	
1370	*Sida rhombifolia*	Scotland Co, N C	6/54	l, s, fl	—	0	0	0	
4783	*Sida spinosa*	Baltimore Co, Md	7/54	l, s, fl, r	—	0	0	0	
		MELASTOMATACEAE							
1023	*Clidemia capitellata*	Tingo Maria, Peru	4/54	l, s	—	0	0	++	+++
1038	*Leandra dichotoma*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	+
1037	*Miconia clathrantha*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	++
1336	*Rhexia alifanus*	Leon Co, Fla	6/54	l, s, fl, fr, r	—	0	0	0	
1335	*Rhexia cubensis*	Leon Co, Fla	6/54	l, s, fl, r	—	0	0	0	
1358	*Rhexia lutea*	Tift Co, Ga	6/54	l, s, fl, r	—	0	0	0	
1298	*Rhexia mariana*	Hanover Co, Va	6/54	l, s, fl	—	0	0	0	
1574	*Rhexia mariana*	Brunswick Co, N C	7/54	l, s, fl, r	—	0	0	0	
		MENISPERMACEAE							
1091	*Cocculus Carolinus*	Clarke Co, Ga	4/54	l	—	0	+	0	+
1110	*Menispermum Canadense*	Monongalia Co, W Va	5/54	l, s	—	0	+++	0	0
		MORACEAE							
1720	*Artocarpus hypargyrea*	C G F	7/54	l, s	—	0	0	0	
1095	*Broussonetia papyrifera*	Clarke Co, Ga	4/54	l	—	0	0	0	0
1289	*Broussonetia papyrifera*	Baltimore Co, Md	6/54	l, s	—	0	0	0	
1116	*Ficus bonplandiana*	C G F	5/54	l, s, fr	—	0	0	0	0
1113	*Ficus callosa*	C G F	4/54	l, s	—	0	0	0	++
1108	*Ficus carica*	C G F	4/54	l, s	—	0	0	0	++
1111	*Ficus cotinifolia*	C G F	4/54	l, s	—	0	0	0	0
1118	*Ficus itcophylla*	C G F	5/54	l, s	—	0	0	0	0
1106	*Ficus kerstingii*	C G F	1/54	l, s	—	0	0	0	+
1115	*Ficus lucescens*	C G F	4/54	l, s, fr	—	0	0	0	+
1112	*Ficus nymphaeaefolia*	C G F	4/54	l, s	—	0	0	0	+
1114	*Ficus odorata*	C G F	4/54	l, s	—	0	0	0	+
1117	*Ficus petiolaris*	C G F	5/54	l, s	—	0	0	0	++
1105	*Ficus payapa*	C G F	4/54	l, s, fr, r	—	0	0	0	0
1119	*Ficus radula*	C G F	5/54	l, s	—	0	0	0	0
1120	*Ficus radulina*	C G F	5/54	l, s	—	0	0	0	0
1109	*Ficus trachyphylla*	C G F	4/54	l, s, fr, r	—	0	0	0	+
1107	*Ficus tsiela*	C G F	4/54	l, s, fr, r	—	0	0	0	+
1110	*Ficus urbaniana*	C G F	4/54	l, s	—	0	0	0	+
1157	*Ficus urbaniana*	C G F	5/54	l, s	—	0	0	0	0
1121	*Ficus urceolaris*	C G F	5/54	l, s	—	0	0	0	0
1251	*Humulus Japonicus*	Johnston Co, N C	6/54	l, s	—	0	0	0	+
4288	*Maclura pomifera*	Baltimore Co, Md	6/54	l, s	—	0	0	0	
1211	*Maclura pomifera*	Wake Co, N C	6/54	l, s	—	0	0	0	0
1046	*Morus alba*	Wake Co, N C	4/54	l, s	—	0	0	0	++
		MYRICACEAE							
4283	*Comptonia peregrina*	Anne Arundel Co, Md	6/54	l, s	—	0	0	++	
4995	*Myrica Carolinensis (Pensylvanica)*	Liberty Co, Fla	8/54	l, s, fr	—	0	0	+++	
4740	*Myrica pusilla*	New Hanover Co, N C	7/54	l, s	—	0	0	+++	

Accession No.	Collection: Species	Collection: Source	Collection: Date Collected	Collection: Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			MYRTACEAE						
4122	*Eucalyptus Algeriensis*	C. G. F.	5/54	l, s	–	0	0	0	0
			NYCTAGINACEAE						
4494	*Mirabilis jalapa*	Craven Co., N. C.	7/54	l, s, fl	–	0	+	0	..
4912	*Mirabilis nyctaginea*	Hammonton, N. J.	8/54	l, s, fl, r	–	0	+	0	..
			NYMPHAEACEAE						
4947	*Nelumbo lutea*	Leon Co., Fla.	8/54	l, s, fl, fr	–	0	0	0	..
4987	*Nuphar advena*	Liberty Co., Fla.	8/54	l, s, fr, r	–	0	+	0	..
4299	*Nymphaea odorata*	Anne Arundel Co., Md.	6/54	l, s, r	–	0	0	0	..
4465	*Nymphaea odorata*	Brunswick Co., N. C.	7/54	l, s, fl, r	–	0	0	0	..
			NYSSACEAE						
4489	*Nyssa aquatica*	Bertie Co., N. C.	7/54	l, s	–	0	0	0	..
4173	*Nyssa sylvatica* var. *biflora*	Brunswick Co., N. C.	6/54	l, s	–	+	0	0	+++
			OLEACEAE						
4050	*Chionanthus Virginicus*	Hancock Co., Ga.	4/54	l	–	0	0	0	+++
4203	*Fraxinus Americana*	Wake Co., N. C.	6/54	l, s	–	++	+	0	++
4176	*Fraxinus Caroliniana*	Brunswick Co., N. C.	6/54	l, s	–	0	0	0	++
4096	*Jasminum humile*	Clarke Co., Ga.	4/54	l	–	0	0	0	++
			ONAGRACEAE						
4664	*Epilobium angustifolium*	Garrett Co., Md.	7/54	l, s, fl, r	–	0	0	+++	..
4821	*Epilobium hirsutum*	Yates Co., N. Y.	8/54	l, s, fl, r	–	0	0	+++	..
4182	*Gaura biennis*	Brunswick Co., N. C.	6/54	l, s, r	–	+++	0	++	0
4961	*Gaura filipes*	Leon Co., Fla.	8/54	l, s, r	–	0	0	0	0 ..
4188	*Jussiaea michauxiana*	New Hanover Co., N. C.	7/54	l, s, r	–	+	0	++	0
4407	*Kneiffia linearis* (*Oenothera fruticosa* var. *linearis*)	Atlantic Co., N. J.	7/54	l, s, fl, r	–	0	0	++	..
4352	*Ludwigia alternifolia*	Benhill Co., Ga.	6/54	l, s, r	–	0	0	+++	..
4566	*Ludwigia linearis*	Brunswick Co., N. C.	7/54	l, s	–	+	0	+++	..
4986	*Ludwigia pilosa*	Liberty Co., Fla.	8/54	l, s, fl, r	–	0	0	+++	..
4459	*Ludwigia suffruticosa*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	++	..
4353	*Ludwigia virgata*	Benhill Co., Ga.	6/54	l, s, fl, r	–	0	0	+++	..
4166	*Oenothera biennis*	Brunwsick Co., N. C.	6/54	l, s, r	–	+	0	+	+
4922	*Trapa natans*	Charles Co., Md.	8/54	fr	–	0	0	0	..
			OPHIOGLOSSACEAE						
4862	*Botrychium Virginianum*	Frederick Co., Md.	8/54	l, s	–	0	0	0	..
			OROBANCHACEAE						
4870	*Conopholis Americana*	Harford Co., Md.	8/54	s, fr	–	0	0	0	..
			OSMUNDACEAE						
4603	*Osmunda cinnamonea*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4856	*Osmunda claytoniana*	Frederick Co., Md.	8/54	l, s, r	–	0	0	0	..
4972	*Osmunda regalis*	McIntosh Co., Ga.	4/54	l	–	0	0	0	0
			OXALIDACEAE						
4785	*Oxalis Europaea f. cymosa*	Baltimore Co., Md.	7/54	l, s, r	–	0	0	0	..
4086	*Osalic stricta*	Clarke Co., Ga.	4/54	l, s	+[a]	0	0	0	0

			PALMAE						
4710	*Acoeloraphe arborescens*	C. G. F.	7/54	l, s	—	0	0	+++	..
4224	*Aeria attenuata*	C. G. F.	6/54	l, s, fr	—	0	0	0	0
4718	*Areca cathcu*	C. G. F.	7/54	l, s, fr	—	0	++	0	..
4380	*Arecastrum romanzoffianum*	C. G. F.	7/54	l, s, fr	—	0	0	0	..
4349	*Butia capitata*	Irwin Co., Ga.	6/54	s, fr	—	0	0	0	..
4973	*Sabal palmetto*	Wakulla Co., Fla.	8/54	l, s	—	0	0	+	..
4886	*Serenoa repens*	Leon Co., Fla.	8/54	l, s	—	0	0	+++	..
			PAPAVERACEAE						
4133	*Chelidenium majus*	Washington Co., Md.	5/54	l, s	—	0	++	0	0
4859	*Sanguinaria Canadensis*	Frederick Co., Md.	8/54	l, s, r	—	0	+++	0	..
			PASSIFLORACEAE						
4330	*Passiflora incarnata*	Wake Co., N. C.	6/54	l, s, fr, r	—	0	+	0	..
			PHYTOLACCACEAE						
4069	*Phytolacca Americana*	McIntosh Co., Ga.	4/54	l	—	0	0	0	+
4276	*Phytolacca Americana*	Baltimore Co., Md.	6/54	l, s	+[a]	0	0	0	..
			PINACEAE						
4403	*Chomaecyparis thyoides*	Atlantic Co., N. J.	7/54	l, s	—	0	0	0	..
4549	*Picea abies*	Macon Co., N. C.	7/54	l, s	—	0	0	+	..
4998	*Pinus caribaea*	Liberty Co., Fla.	8/54	l, s	—	0	0	+	..
4440	*Pinus serotina*	Brunswick Co., N. C.	7/54	l, s, fr	—	0	0	0	..
4514	*Pinus strobus*	Haywood Co., N. C.	7/54	l, s	—	0	0	0	..
4452	*Taxodium ascendens*	Brunswick Co., N. C.	7/54	l, s, fr	—	+	0	+	..
4550	*Thuja occidentalis*	Transylvania Co., N. C.	7/54	l, s	—	0	0	++	..
4502	*Tsuga Canadensis*	Haywood Co., N. C.	7/54	l, s	—	0	0	0	..
4662	*Tsuga Canadensis*	Garrett Co., Md.	7/54	l, s	—	0	0	0	..
			PIPERACEAE						
4001	*Peperomia sp.*	Tingo Maria, Peru	4/54	l, r	—	0	0	0	+
4003	*Piper sp.*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	+++
			PLANTAGINACEAE						
4259	*Plantago aristata*	Wake Co., N. C.	6/54	l, s, fl	—	0	0	0	..
4396	*Plantago aristata*	Baltimore Co., Md.	7/54	l, s, fr, r	—	0	0	0	..
4055	*Plantago Virginica*	McIntosh Co., Ga.	4/54	l	—	0	0	0	++
			PLATANACEAE						
4210	*Platanus occidentalis*	Wake Co., N. C.	6/54	l, s	—	0	0	0	+
			PLUMBAGINACEAE						
4773	*Limonium Carolinianum*	Brunswick Co , N. C.	7/54	l, s, r	—	0	0	+	..
			POLEMONIACEAE						
4562	*Phlox Carolina* var. *triflora*	Macon Co., N. C.	7/54	l, s, fl, r	—	+	0	0	..
4144	*Phlox divaricata* (var. *Canadensis*)	Monongalia Co., W. Va.	5/54	l, s, fl, r	—	0	0	0	+
4316	*Phlox maculata*	Durham Co., N. C.	6/54	l, s, fl, r	—	0	0	0	..
			POLYGALACEAE						
4914	*Polygala cruciata*	Atsion, N. J.	8/54	l, s, fl, r	—	0	0	0	.
4181	*Polygala lutea*	Brunswick Co., N. C.	6/54	l, s, r, fl	—	++	0	0	+

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1390	*Polygala mariana*	Caroline Co Va	7/54	l, s	+[a]	0	0	0	
4431	*Polygala ramosa*	Brunswick Co, N C	7/54	l, s, fl, r	−	0	0	0	
		POLYGONACEAE							
1798	*Eriogonum gracile*	Dripping Springs, Calif.	7/54	w	−	+	0	0	
4360	*Eriogonum tomentosum*	Emanuel Co, N C	6/54	l, s, fl, r	−	0	0	++	
4820	*Fagopyrum esculentum* (*F sagittatum*)	Yates Co, N C	8/54	l, s, fl, r	−	0	0	0	
4368	*Polygonella Americana*	Richmond Co, Ga	6/54	l, s, fl, r	−	0	0	0	
4984	*Polygonella gracilis*	Liberty Co, Fla	8/54	s, r	−	0	0	++	
4730	*Polygonum arifolium*	New Hanover Co, N C	7/54	l, s	−	0	0	+	
4869	*Polygonum cuspidatum*	Harford Co, Md	8/54	l s, fl	−	0	0	+	
1318	*Polygonum Pensylvanicum*	Wake Co, N C	6/54	l, s	−	0	0	0	
1899	*Thysanella fimbriata*	Franklin Co, Fla	8/54	l, s, fl, r	−	0	0	++	
4861	*Tovara Virginiana*	Frederick Co, Md	8/54	l, s, fl, r	−	0	0	0	
		POLYPODIACEAE							
1857	*Adiantum pedatum*	Frederick Co, Md	8/54	l, s, r	−	0	0	+	
4669	*Athyrium thelypteroides*	Baltimore Co, Md	7/54	l, s	−	0	0	0	
4548	*Dennstaedtia punctilobula*	Transylvania Co, N C	7/54	l, s, r	−	0	0	0	
1858	*Dryopteris hexagonoptera*	Frederick Co, Md	8/54	l, s r	−	0	0	0	
4677	*Dryopteris marginalis*	Harford Co, Md	7/54	l, s, r	−	0	0	0	
1213	*Dryopteris noveboracensis*	Wake Co, N C	6/54	l	−	0	+	0	0
1536	*Onoclea sensibilis*	Buncombe Co, N C	7/54	l, s	−	0	0	0	
4655	*Polypodium Virginianum*	Monongalia Co, W Va	7/54	l, s, r	−	0	0	0	
4438	*Woodwardia areolata*	Brunswick Co, N C	7/54	l, s	−	0	0	0	
		PONTEDERIACEAE							
4071	*Pontederia cordata*	McIntosh Co, Ga	4/54	l	−	0	0	0	0
		PORTULACACEAE							
4731	*Portulaca oleracea*	New Hanover Co, N C.	7/54	l, s, fl, r	−	0	0	0	
4784	*Portulaca oleracea*	Baltimore Co, Md	7/54	l s, fl, r	−	0	0	0	
		POTAMOGETONACEAE							
4927	*Potamogeton cf perfoliatus*	Charles Co, Md	8/54	l, s, fl	−	0	0	0	
		PRIMULACEAE							
4601	*Lysimachia quadrifolia*	Tucker Co, W Va	7/54	l, s, r	−	+	0	0	
4564	*Lysimachia quadrifolia*	Macon Co, N C	7/54	l, s, fl, r	−	+	0	0	
4867	*Lysimachia vulgaris*	Harford Co, Md	8/54	l, s, fl, r	−	0	0	0	
		RANUNCULACEAE							
4606	*Anemone Virginiana*	Raleigh Co, W Va	7/54	l, s, fl r	+[b]	0	0	0	
1129	*Anemonella thalictroides*	Washington Co, Md	5/54	l, s	−	0	+	0	++
1139	*Cimicifuga racemosa*	Monongalia Co, W Va.	5/54	l, s, r	−	0	0	0	0
4111	*Clematis Virginiana*	Monongalia Co, W Va	5/54	l, s	−	0	0	0	+
4149	*Hepatica acutiloba*	Preston Co, W Va	5/54	l, s, r	−	0	0	0	+
4801	*Paeonia brownii*	Dripping Springs, Calif	7/54	w	−	+	0	+++	
4106	*Thalictrum polygamum*	Atlantic Co, N J	7/54	l, fl, r	−	0	++	0	
1542	*Xanthorrhiza simplicissima*	Henderson Co, N C	7/54	l, s, r	−	0	+	0	

	RHAMNACEAE								
4749	*Berchemia scandens*	Carteret Co., N. C.	7/54	l, s	—	+	0	+	..
4223	*Ceanothus Americanus*	Wake Co., N. C.	6/54	l, s, fl	—	0	++	++	0
4824	*Rhamnus catharticus*	Yates Co., N. Y.	8/54	l, s, fr	—	0	0	0	..
4622	*Rhamnus lanceolatus*	Wayne Co., Ky.	7/54	l, s	—	0	0	0	..
	ROSACEAE								
4792	*Adenostoma sparsifolium*	Dripping Springs, Calif.	7/54	l	—	0	0	++	..
4531	*Agrimonia gryposepala*	Buncombe Co., N. C.	7/54	l, s, fl, r	—	0	0	0	..
4309	*Agrimonia parviflora*	Chatham Co., N. C.	6/54	l, s, r	—	0	0	0	..
4195	*Aronia arbutifolia*	Sampson Co., N. C.	6/54	l, s	—	+	0	0	0
4603	*Aruncus dioicus*	Clay Co., W. Va.	7/54	l, s, fl, r	—	0	0	0	..
4670	*Chaenomeles lagenaria*	Baltimore Co., Md.	7/54	l, s, fr	—	0	0	0	..
4996	*Chrysobalanus oblongifolius*	Liberty Co., Fla.	8/54	l, s, fr	—	0	0	+++	..
4815	*Geum aleppicum* var. *strictum*	Yates Co., N. Y.	8/54	l, s, fl, r	—	0	0	++	..
4626	*Gillenia stipulata*	Hopkins Co., Ky.	7/54	l, s, fl, r	—	0	0	+	..
4602	*Gillenia trifoliata*	Randolph Co., W. Va.	7/54	l, s, fl	—	0	0	0	..
4394	*Physocarpus opulifolius*	Baltimore Co., Md.	7/54	l, s, fl	—	0	0	0	..
4812	*Potentilla recta*	Yates Co., N. Y.	8/54	l, s, fl, r	—	0	0	+	..
4765	*Prunus ilicifolia*	Aguanga, Calif.	8/54	l	—	0	0	+++	..
4944	*Prunus maritima*	Sussex Co., Del.	8/54	l, s	—	0	0	+++	..
4560	*Prunus Pensylvanica*	Macon Co., N. C.	7/54	l, s	—	0	0	+	..
4823	*Prunus Virginiana*	Yates Co., N. Y.	8/54	l, fr, r	—	0	0	0	..
4145	*Pyrus (Malus) coronaria*	Taylor Co., W. Va.	5/54	l, s	—	+++	0	++	++
4943	*Pyrus (Malus) sp.*	Sussex Co., Del.	8/54	l, s	—	0	0	+++	..
4479	*Rosa Carolina*	Elizabeth City Co., Va.	7/54	l, s	—	+	0	+++	..
4327	*Rubus argutus*	Wake Co., N. C.	6/54	l, s, fl	—	0	0	0	..
4767	*Rubus cuneifolius*	Carteret Co., N. C.	7/54	l, s	—	0	0	++	..
4523	*Rubus idaeus* var. *Canadensis*	Buncombe Co., N. C.	7/54	l, s	—	0	0	0	..
4832	*Rubus idaeus* var. *strigosus*	Cayuta Lake, N. Y.	8/54	l, s	—	0	0	0	..
4508	*Rubus odoratus*	Haywood Co., N. C.	7/54	l, s, fl	—	0	0	0	..
4661	*Rubus odoratus*	Garrett Co., Md.	7/54	l, s	—	0	0	0	..
4667	*Rubus phoenicolasius*	Baltimore Co., Md.	7/54	l, s, fl	—	0	0	+	..
4780	*Sanguisorba Canadensis*	Baltimore Co., Md.	7/54	l, s	—	0	0	+++	..
4837	*Sorbaria sorbifolia*	Bradford Co., Pa.	8/54	l, s, fl, r	—	0	0	0	..
4703	*Spiraea tomentosa*	Wicomico Co., Md.	7/54	l, s, r	—	0	0	0	..
	RUBIACEAE								
4384	*Adina cordifolia*	C. G. F.	7/54	l, s	—	0	0	0	..
4383A	*Alibertia edulis*	C. G. F.	7/54	l	—	0	0	0	..
4383B	*Alibertia edulis*	C. G. F.	7/54	s	—	0	0	+	..
4383C	*Alibertia edulis*	C. G. F.	7/54	fr	—	0	0	0	..
4062	*Cephalanthus occidentalis*	McIntosh Co., Ga.	4/54	l	—	0	0	+	+
4004	*Chiococca sp.*	Tingo Maria, Peru	4/54	l, s	—	++	0	0	+
4305	*Diodia teres*	Caroline Co., Va.	6/54	l, s, r	—	0	0	0	..
4360	*Diodia teres*	Richmond Co., Ga.	6/54	l, s, r	+[a]	0	0	0	..
4448	*Diodia Virginiana*	Onslow Co., N. C.	7/54	l, s, r	—	0	0	0	..
4537	*Galium latifolium*	Buncombe Co., N. C.	7/54	l, s, fl, r	—	+	0	0	..
4834	*Galium verum*	Mecklenburg, N. Y.	8/54	l, s, fl, r	—	0	0	0	..
4033	*Gonzalagunia bunchosioides*	Tingo Maria, Peru	4/54	l, s, b	—	0	0	+	++
4012	*Hillia parasitica*	Tingo Maria, Peru	4/54	l, s	—	0	0	0	+

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			RUBIACEAE						
4635	*Houstonia nigricans*	Bullitt Co., Ky.	7/54	l, s, fl	−	++	0	+++	. .
4348	*Pinckneya pubens*	Tift Co., Ga.	6/54	l, s, fr	−	0	+	0	. .
4267	*Richardia scabra*	Brunswick Co., N. C.	6/54	l, s, r	−	0	0	0	. .
			RUTACEAE						
4714	*Atalantia citroides*	C. G. F.	7/54	l, s, fr	−	0	0	0	. .
4152	*Citrus limon*	Chico, Calif.	5/54	l, s	+[a]	+	0	0	+
			SALICACEAE						
4473	*Populus alba*	Brunswick Co., N. C.	7/54	l, s	−	0	0	0	. .
4497	*Populus heterophylla*	Bertie Co., N. C.	7/54	l, s	−	0	0	0	. .
4841	*Populus tremuloides*	Wayne Co., Pa.	8/54	l, s	−	0	0	+	. .
4237	*Salix discolor*	Durham Co., N. C.	6/54	l, s	−	0	0	0	0
4671	*Salix discolor*	Baltimore Co., Md.	7/54	l, s	−	0	0	0	. .
			SANTALACEAE						
4136	*Comandra umbellata*	Washington Co., Md.	5/54	l, s	−	0	0	0	0
4541	*Pyrularia pubera*	Henderson Co , N. C.	7/54	l, s, fr	−	0	0	0	. .
			SAPINDACEAE						
4005	*Cardiospermum sp.*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	+++
4125	*Sapindus saponaria*	C. G. F.	5/54	l, s	−	0	0	0	++
			SARRACENIACEAE						
4081	*Sarracenia flava*	Bulloch Co., Ga.	4/54	l	−	0	0	0	0
4294	*Sarracenia purpurea*	Anne Arundel Co., Md.	6/54	l, s, r	−	+	0	0	. .
			SAPOTACEAE						
4378	*Lucuma ovata*	C. G. F.	7/54	l, s	−	0	0	++	. .
			SAURURACEAE						
4064	*Saururus cernuus*	McIntosh Co., Ga.	4/54	l	−	0	0	0	+
			SAXIFRAGACEAE						
4509	*Astilbe biternata*	Haywood Co., N. C.	7/54	l, s, fl, r	−	0	0	+	. .
4087	*Decumaria barbara*	Clarke Co., Ga.	4/54	l	−	0	0	0	0
4607	*Heuchera Americana*	Wycoming Co., W. Va.	7/54	l, s, fl, r	−	0	0	+++	. .
4534	*Hydrangea arborescens*	Buncombe Co., N. C.	7/54	l, s, fl	−	0	0	0	. .
4513	*Hydrangea radiata*	Haywood Co., N. C.	7/54	l, s, fl	−	0	0	0	. .
4132	*Saxifraga Virginiensis*	Washington Co., Md.	5/54	l, s, fl, r	−	0	0	0	0
4457	*Itea Virginica*	Brunswick Co., N. C.	7/54	l, s, fl	−	0	0	0	. .
			SCROPHULARIACEAE						
4217	*Aureolaria pectinata*	Wake Co., N. C.	6/54	l, s, r	−	0	0	0	0
4310	*Aureolaria Virginica*	Chatham Co., N. C.	6/54	l, s, r	−	0	0	0	. .
4460	*Bacepa Caroliniana*	Brunswick Co., N. C.	7/54	l, s, r	−	0	0	0	. .
4797	*Cordylanthus filifolius*	Dripping Springs, Calif.	7/54	l	−	0	++	++	. .
4779	*Digitalis purpurea*	Baltimore Co., Md.	7/54	l, s, fl, r	−	0	0	0	. .
4399	*Gerardia pedicularia*	Atlantic Co., N. J.	7/54	l, s, r	−	0	0	0	. .
4391	*Gerardia Virginica*	Hanover Co., Va.	7/54	l, s, fl	+[a]	0	0	0	. .

4991	*Macranthera flammea*	Liberty Co , Fla	8/54	l, s	—	0	0	0	.
4400	*Melampyrum lineare*	Atlantic Co , N J	7/54	l, s, fl, r	—	0	0	0	. .
4610	*Mimulus ringens*	Mingo Co , W Va	7/54	l, s, r	—	0	0	0	. .
4098	*Paulownia tomentosa*	Clarke Co , Ga	4/54	l	—	0	0	0	0
4100	*Paulownia tomentosa*	Clarke Co , Ga	4/54	l	—	0	0	0	+
4515	*Pedicularis Canadensis*	Haywood Co , N C	7/54	l, s	—	0	0	0	
4260	*Penstemon laevigatus*	Wake Co , N C	6/54	l, s, fl, r	—	0	0	0	
4350	*Penstemon multiflorus*	Tift Co , Ga	6/54	l, s, fl, r	—	0	0	0	
4956	*Seymeria cassioides*	Leon Co , Fla	8/54	l, s, r	—	0	0	+	
4591	*Verbascum blattaria*	Carroll Co , Md	7/54	l, s, fl, r	+[a]	0	0	0	.
4866	*Veronica anagallis-aquatica*	Harford Co , Md	8/54	l, s, fl, r	—	0	0	0	. .
		SELAGINELLACEAE							
4517	*Selaginella rupestris*	Transylvania Co , N C	7/54	l, s	—	+	0	0	.
		SIMAROUBACEAE							
4264	*Ailanthus altissima*	Brunswick Co , N C	6/54	l, s	—	0	0	0	.
		SOLANACEAE							
4708	*Cestrum nocturnum*	C G F.	8/54	l, s	+[a]	0	0	0	.
4709	*Cestrum parqui*	C G F.	8/54	l, s	—	0	+	+++	.
4379A	*Cestrum parqui*	C G F	7/54	l	—	0	0	++	
4379B	*Cestrum parqui*	C G F.	7/54	tw	—	0	0	0	
4371A	*Cestrum sp*	C G F.	8/54	l, s	+[a]	0	0	0	
4713B	*Cestrum sp*	C G F.	8/54	fr	+[b]	0	0	0	
4485	*Datura stramonium*	Nansemond Co , Va	7/54	l, s	—	0	++	0	
4641	*Datura stramonium*	Bullitt Co , Ky	7/54	l, s	—	0	+	0	+++
4097	*Lycium halimifolium*	Clarke Co , Ga	4/54	l	—	0	0	0	
4769	*Physalis maritima*	Brunswick Co , N C	7/54	l, s, fr, r	—	0	++	0	+++
4054	*Physalis viscosa*	McIntosh Co , Ga	4/54	l	—	0	0	0	
4328	*Solanum Carolinense*	Wake Co , N C	6/54	l, s, fr, r	—	0	0	0	.
4134	*Solanum dulcamara*	Washington Co , Md	5/54	l, s	—	0	+	0	0
4738	*Solanum nigrum*	New Hanover Co , N C	7/54	l, s, fl, r	+[b]	0	+	0	.
		SPARGANIACEAE							
4583	*Sparganium Americanum*	Brunswick Co , N C.	7/54	l, s, fl, r	—	0	0	0	
		STAPHYLEACEAE							
4674	*Staphylea trifolia*	Harford Co , Md.	7/54	l, s, fl, r	—	0	0	0	. .
		STERCULIACEAE							
4123	*Abroma augusta*	C G F.	6/54	l, s, fr	—	0	0	0	0
		STYRACACEAE							
4084	*Halesia Carolina*	Clarke Co , Ga.	4/54	l	—	0	0	0	++
4556	*Halesia Carolina*	Macon Co , N C	7/54	l, s, fr	—	0	0	0	
4512	*Styrax grandifolia*	Haywood Co , N C	7/51	l, s	—	0	0	0	.
		SYMPLOCACEAE							
4433	*Symplocos tinctoria*	Brunswick Co , N. C.	7/54	l, s	+[a]	+	0	0	
		TAMARICACEAE							
4469	*Tamarix gallica*	Brunswick Co , N C.	7/54	l, s	—	+	0	+++	

Accession No.	Collection: Species	Collection: Source	Collection: Date Collected	Collection: Plant Part	Hemolysis test	Flavonoids	Alkaloids	Tannins	Sterols
			TILIACEAE						
4676	*Tilia Americana*	Harford Co., Md.	7/54	l, s, fr	–	0	0	+	. .
4313	*Tilia Floridana*	Chatham Co., N. C.	6/54	l, s, fl	–	0	0	0	. .
4024	*Triumfetta sp.*	Tingo Maria, Peru	4/54	l, s, fr	–	0	0	0	+++
			TURNERACEAE						
4953	*Piriqueta Caroliniana*	Leon Co., Fla.	8/54	l, s, fl, r	–	0	0	0	. .
			TYPHACEAE						
4772	*Typha glauca*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	++	++	. .
4431	*Typha latifolia*	Brunswick Co., N. C.	7/54	l, s, fr, r	–	0	0	0	. .
			ULMACEAE						
4475	*Celtis laevigata*	Elizabeth City Co., Va.	7/54	l, s	–	0	0	0	. .
4645	*Celtis occidentalis*	Mason Co., Ky.	7/54	l, s, fl, r	–	0	0	0	. .
4621	*Ulmus alata*	Wayne Co., Ky.	7/54	l, s	–	0	0	+	. .
4202	*Ulmus Americana*	Wake Co., N. C.	6/54	l, s	–	+	0	0	+
			UMBELLIFERAE						
4555	*Angelica triquinata*	Macon Co., N. C.	7/54	l, s, fl, r	–	0	0	0	. .
4262	*Angelica villosa*	Johnston Co., N. C.	6/54	l, s, fl, r	–	0	0	0	. .
4236	*Cicuta maculata*	Durham Co., N. C.	6/54	l. s	–	0	0	0	0
4458	*Centella erecta*	Brunswick Co., N. C.	7/54	l, s, r	–	0	0	0	. .
4833	*Conioselinum Chinense*	Mecklenburg, N. Y.	8/54	l, s, fl	–	0	0	0	. .
4754	*Eryngium aquaticum*	Carteret Co., N. C.	7/54	l, fl, r	–	0	+	0	. .
4341	*Eryngium prostratum*	Leon Co., Fla.	6/54	l, s, fl	–	0	0	0	. .
4369	*Eryngium yuccifolium*	Emanuel Co., Ga.	6/54	l, s, fl	+[a]	0	0	0	. .
4519	*Levisticum officinale*	Buncombe Co., N. C.	7/54	l, s, fl, fr	–	0	0	0	. .
4324	*Ligusticum Canadense*	Orange Co., N. C.	6/54	l, s, fl	–	0	+	0	. .
4860	*Osmorhiza claytoni*	Frederick Co., Md.	8/54	l, s, r	–	0	0	0	. .
4976	*Oxypolis filiformis*	Wakulla Co., Fla.	8/54	l, s, r	–	0	0	0	. .
4672	*Petroselinum crispum*	Baltimore Co., Md.	7/54	l, s, r	+[a]	0	0	0	. .
4167	*Ptilimnium capillaceum*	Brunswick Co., N. C.	6/54	l, s, fl	–	+	0	0	0
4333	*Sanicula Marilandica*	Wake Co., N. C.	6/54	l, s, r	+[a]	0	+	0	. .
4755	*Sium suava*	Carteret Co., N. C.	7/54	l, s, fl	–	0	0	0	. .
4642	*Torilis Japonica*	Anderson Co., Ky.	7/54	l, s, r	–	0	0	0	. .
			URTICACEAE						
4683	*Boehmeria cylindrica*	Dorchester Co., Md.	7/54	l, s, fl, r	–	0	0	0	. .
4270	*Boehmeria drummondii*	Brunswick Co., N. C.	6/54	l, s, r	–	0	0	0	. .
4150	*Laportea Canadensis*	Preston Co., W. Va.	5/54	l, s, r	–	0	0	0	++
4673	*Pilea pumila*	Harford Co., Md.	7/54	l, s, r	–	0	0	0	. .
4865	*Urtica dioica*	Harford Co., Md.	8/54	l, s, fl	+[a]	0	0	0	. .
4814	*Urtica gracilis*	Yates Co., N. Y.	8/54	l, s, r	–	0	0	0	. .
			VERBENACEAE						
4068	*Callicarpa Americana*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4470	*Lantana camara*	Brunswick Co., N. C.	7/54	l, s, fr	–	0	0	0	. .
4058	*Lippia nodiflora*	McIntosh Co., Ga.	4/54	l	–	0	0	0	+
4455	*Verbena bonariensis*	Brunswick Co , N. C.	7/54	l, s, fl	–	0	0	0	. .

4085	*Verbena rigida*	Clarke Co, Ga.	4/54	l, s	−	0	0	0	+
4619	*Verbena simplex*	Pulaski Co, Ky	7/54	l, s, fl	−	0	0	0	
4248	*Verbena urticifolia*	Wake Co, N C	6/54	l, s	−	0	0	0	++
		VIOLACEAE							
4015	*Viola papilionacea*	Wake Co, N. C	4/54	l, s	−	0	0	0	+
4135	*Viola pedata*	Washington Co, Md.	5/54	l, s, fl, r	−				
4143	*Viola striata*	Monongalia Co, W Va.	5/54	l, s, r	+[a]	0	0	0	+
		VITACEAE							
4269	*Ampelopsis arborea*	Brunswick Co, N C.	6/54	l, s	−	+	0	++	
4022	*Cissus sp.*	Tingo Maria, Peru	4/54	l, s	−	0	0	0	+++
4208	*Parthenocissus quinquifolia*	Wake Co, N C	6/54	l, s, r	−	0	0	0	+
4412	*Vitis rotundifolia*	Brunswick Co, N C	7/54	l, s, fr	−	0	0	0	
4206	*Vitis vulpina*	Wake Co, N. C.	6/54	l, s	−	0	0	+	++
		XYRIDACEAE							
4573	*Xyris difformis*	Brunswick Co, N. C.	7/54	l, s, fl, r	−	+	0	0	.
		ZINGIBERACEAE							
4711	*Phaeomeria speciosa*	C. G. F	7/54	l, s, r	−	0	0	0	
		ZYGOPHYLLACEAE							
4743	*Tribulus terrestris*	New Hanover Co., N C.	7/54	l, s, fl	+[b]	0	0	0	

[a] No steroidal sapogenins found [b] See text for steroidal sapogenins found

CORRECTIONS ON ACCESSIONS

CORRECTIONS ON SECOND 1,000 ACCESSIONS (4)

Page	Acces No	Should Read
14	1997	*Dioscorea spiculiflora*
14	1999	*Dioscorea floribunda*
27	1709	*Cereocarpus*

CORRECTIONS ON THIRD 1,000 ACCESSIONS (6)

Page	Acces. No	Should Read
4	2328	*Agave lecheguilla*, var. *Dioscorea*
13	2249	*Mexicana*

CORRECTIONS ON FOURTH 1 000 ACCESSIONS (7)

Page	Acces No	Should Read
655	3341	*Agave deserti*
656	3104	*Agave roezliana*
665	3112	*Dioscorea floribunda*
665	3115	*Dioscorea floribunda*
665	3129	*Dioscorea plumifera*
665	3168	*Dioscorea floribunda*
665	3174	*Dioscorea cf. floribunda*
666	3358	*Dioscorea bulbifera*
666	3359	*Dioscorea bulbifera*
666	3360	*Dioscorea bulbifera*
666	3369	*Dioscorea Mexicana*
668	3547	*Dioscorea floribunda*
668	3551	*Dioscorea floribunda*
669	3736	*Dioscorea convolvulacea*
669	3737	*Dioscorea convolvulocea*
669	3738	*Dioscorea convolvulacea*
669	3739	*Dioscorea densiflora*
669	3742	*Dioscorea n sp*
669	3745	*Dioscorea convolvulacea*
669	3746	*Dioscorea convolvulacea*
669	3748	*Dioscorea Mexicana*
669	3749	*Dioscorea convolvulacea*
669	3751	*Dioscorea galeottiana*
669	3755	*Dioscorea lobata* var *morelosana*
670	3758	*Dioscorea convolvulacea*
670	3849	*Acalypha sp*
670	3950	*Acalypha sp*
670	3057	*Alchorneo*
673	3586A	*Yucca filamentosa*
678	3276	*In Phytolaccaceae*
678	3681	*Polystichum acrostichoides*
679	3118	*Poterium sanguisorba*

Flavonoids were found in 96 species, of which at least half seem to be new to the record

Alkaloids were found in 86 species, an incidence of about 9% of the total This is higher than noted in previous series, due largely to the change in technique mentioned above Most of the species were wild plants growing in the eastern United States Some 45 species are new to the record.

Tannins were again conspicuously absent from 150 species of Compositae There were no noteworthy occurrences of tannin except in *Rhus*, and here it is expected

Unsaturated sterols have proved to be so ubiquitous that the test for them was made on only a portion of the accessions

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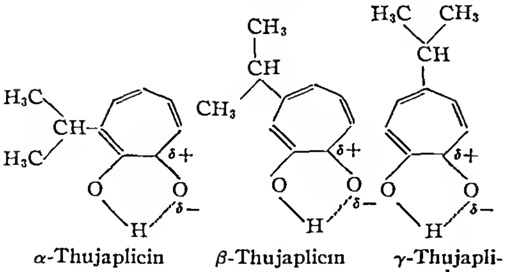
A Pharmacological Study of Gamma-Thujaplicin*

By JOHN E. HALLIDAY

Gamma-thujaplicin (2-hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one), a tropolone derivative occurring in western red cedar, has been studied pharmacologically in the form of its sodium salt (T-Na). It has been found to exert a combination of stimulant and depressant actions on the mammalian central nervous system and only depression in frogs. Stimulant effects, in the form of convulsions shown to be of cerebral origin, are evident only in untreated animals. Certain signs of depression can also be observed in untreated animals while further depressant actions are revealed only in animals which have been treated with narcotic drugs. T-Na reduces the responses of isolated frog skeletal muscle and antagonizes the action of some stimulant drugs on isolated intestinal muscle.

GAMMA-THUJAPLICIN (2-hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one) is one of three isomeric isopropyltropolones which occur in the heartwood of western red cedar (*Thuja plicata* D Don). The term tropolone is used to designate the seven-membered carbon ring compound 2-hydroxy-2,4,6-cycloheptatrien-1-one, derivatives of which are relatively rare in nature. The thujaplicins, which were the first naturally-occurring compounds to be characterized conclusively as monocyclic tropolones (1) and are the simplest of the natural tropolone derivatives, have the following structures:



α-Thujaplicin β-Thujaplicin γ-Thujaplicin

Other substances found in nature containing the tropolone structure include the mold metabolites stipitatic and puberulic acids, and the alkaloid colchicine (2).

The chemistry of the thujaplicins and other tropolone derivatives has been studied quite extensively (2, 3). Antimicrobial studies of the thujaplicins have shown that all of the isomers possess antifungal and antibacterial properties and it is interesting to note that their antifungal activity has been given as the reason for the durability of cedar wood (4, 5, 6). The only pharmacological studies of thujaplicins which have been reported appear to be those by Lee (7, 8, 9) and other Japanese workers (10, 11, 12) on β-thujaplicin. This isomer is commonly called hinokitiol by the Japanese, who obtain it from the tree known as Formosan hinoki (*Chamaecyparis taiwensis*, Masamune et Suzuki—*Chamaecyparis obtusa*, Sieb et Zucco F. *Formosana*, Hayata) Lee found that its actions were largely depressant in nature though low concentrations may stimulate some organs, e g, isolated heart and rabbit uterus. It was suggested by Katsura (13), who has summarized Lee's work, that β-thujaplicin is a nerve-muscle poison related in its actions to camphor and thujone, and other terpene compounds Since there is a lack of pharmacological information on γ-thujaplicin, and since supplies of this isomer were available, the present investigation was undertaken.

Gamma-thujaplicin was obtained as a white crystalline substance melting between 79 and 80° As the free substance is only slightly soluble in water, the sodium salt was used in the experiments described here This was precipitated from a concentrated solution of γ-thujaplicin in alcohol by the addition of a freshly prepared alcoholic solution of sodium ethylate.

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The author is indebted to Dr J G Gardner of the Vancouver Laboratory, Forest Products Laboratories of Canada, for supplies of γ thujaplicin used in this study, and to Mr J G Moir for assistance in preparing photographs
Appreciation is expressed to Abbott Laboratories Ltd, Ciba Company Ltd, Montreal, and to E R Squibb & Sons of Canada Ltd, for supplying some of the drugs used in this investigation

The precipitate was collected and dried, then powdered and washed with successive portions of anhydrous ether, and again dried, to give a yellow powder. γ-Thujaplicin sodium (T-Na) dissolves readily in water to form alkaline solutions, the pH of a 1% solution being 8.4.

EXPERIMENTAL PROCEDURES AND RESULTS

Effects in Intact Animals.—Most of the observations of the effects of T-Na in intact, conscious animals were made following injection of a 1 or 5% aqueous solution into Swiss albino mice, Sprague-Dawley rats, and rabbits. Six cats and two dogs were also injected.

In all five species of mammals T-Na produced convulsions of a mixed clonic and tonic type. Doses producing convulsions in the various species, expressed as mg. per Kg. of body weight, were: mice and rats, i. p., 75 to 100, oral, 150 or more; rabbits i. v., 25 to 40; cats i. p., 50 to 70; dogs i. v., 30 and 40. Depending on the dose, one to several convulsive seizures occurred, lasting from five to fifteen seconds and recurring at intervals which varied from fifteen to ninety seconds. The convulsions were preceded by a brief period of ataxia and followed by symptoms of depression which included slowing of reflexes, decrease in muscle tone and in voluntary movement, and absence of certain placing reactions, such as the dorsal contact reaction, the chin reaction, and the edge reflex. During this period the animals would lie either prone or on one side, with limbs extended and somewhat flaccid. No attempts to move or regain a normal position were made. When the larger animals were placed on their feet they collapsed again to a lying position. Righting reflexes were reduced but not abolished. In rodents, a decrease in respiratory rate, usually accompanied by an increase in respiratory depth, was also observed. In animals in which these effects were marked, the appearance was one of slow, labored breathing. The duration of the postconvulsive depression varied with the dose, most signs of depression disappearing within twenty minutes following the last seizure. In rodents, respiration was the last function to return to normal, often taking one to three hours. When death occurred from the larger doses, it was always during the post-convulsive depressive stage following a period of gradually declining respiratory activity, and never during the convulsive seizure as is the case with such convulsive agents as metrazol.

T-Na was also administered to frogs, either by way of the dorsal lymph sac or via the abdominal vein. Here the effect of T-Na was one of depression only. Doses of 50 to 500 mg./Kg. caused the frogs to slump to a prone position with reduced reflex activity and respiratory movements, but no initial stimulant effect was observed. Death occurred in all frogs which received more than 100 mg./Kg.

Acute Toxicity and Convulsive Dose in Mice.—The intraperitoneal LD_{50} and CD_{50} (50% convulsive dose) were determined in Swiss albino mice which had been fasted for twelve hours. For each determination seven groups of 10 mice each were used. All deaths were recorded which occurred during a period of four days following administration of T-Na. The mice in which death was delayed were in a state of depression, following convulsions, which deepened progressively, terminating in respiratory paralysis. There were no deaths after the fourth day. When calculated according to the graphic method of Miller and Tainter (14) the intraperitoneal LD_{50} was found to be 162 $\pm$ 3.2 mg./Kg. and the intraperitoneal CD_{50} 73.5 $\pm$ 1.8 mg./Kg.

Site of Convulsive Action.—Six Sprague-Dawley rats, after ligation of the common carotids under ether anesthesia were decerebrated according to methods described in the literature (15, 16). Five other rats, with the common carotids ligated but otherwise intact, served as controls. After allowing at least two hours to elapse for elimination of ether, T-Na was administered intraperitoneally. Doses from 150 to 300 mg./Kg. failed to elicit convulsions in any of the decerebrate rats though convulsions were produced in all of the control rats, including two rats in which the dose was only 100 mg./Kg. Decerebrate rigidity, which was present to a marked degree in three of the decerebrate rats and to a lesser degree in the others, was not significantly altered by T-Na. These results, which indicate a cerebral site for the convulsant action of T-Na, were supported by observations in four decerebrate cats used in later experiments for reflex studies. No convulsive activity was produced in any of these cats by intravenous doses of from 40 to 75 mg./Kg.

Interaction of T-Na with Hypnotic Agents.—The possibility that the convulsive action of T-Na would antagonize the action of hypnotic drugs was investigated by determining its effect on the sleeping times of mice following administration of pentobarbital sodium, thiopental sodium, and chloral hydrate. For comparison with a known analeptic agent, the effect of metrazol on the sleeping time was also determined. Ten mice were used at each dosage level. T-Na was injected intraperitoneally immediately following administration of the barbiturates and ten to fifteen minutes following administration of chloral hydrate. Sleeping time was taken as the period from the time the mice first could be placed on their backs until they could turn from their backs to an upright position. The results, shown in Table I, indicate that T-Na does not possess analeptic properties. In fact, an apparent prolongation of sleeping times occurred in some groups when T-Na was administered.

Action on Spinal Reflexes.—The possibility that muscle hypotonia seen in animals which had been given T-Na was due to depression of interneuronal transmission of impulses was investigated by determining the effect of T-Na on the flexion reflex in the cat. Six experiments were performed on cats lightly anesthetized with 0.75 cc. to 0.80 cc. of Dial[1] with urethane per Kg. Four experiments were performed on decerebrate cats and six on spinal cats, prepared under ether anesthesia according to methods described by Burn (17). Artificial respiration was maintained by means of a Palmer variable stroke Ideal respirator pump. The method of recording the flexion reflex was a modification of those described by Slater, *et al.* (18), and Kaada (19) with reflex contractions of the anterior tibealis

[1] Trademark of Ciba Co. Ltd.

TABLE I.—EFFECT OF T-Na ON DURATION OF SLEEPING TIMES PRODUCED BY CENTRAL DEPRESSANTS IN MICE

Depressant Dose and Route	T-Na, mg./Kg., i. p	Metrazol, mg./Kg., i. p.	No. of Mice	Sleeping Time, min. ± S. D.
Thiopental Na,	..	..	10	14.5 ± 11.5
50 mg./Kg., i.p.	50	..	10	21.2 ± 14.8
	100	..	10	54.3 ± 22.7
	200	..	6[a]	245.8 ± 22.9
	..	50	10	3.8 ± 3.5[b]
Pentobarbital Na	..	..	10	113.3 ± 47.6
75 mg./Kg., i.v.	50	..	10	122.6 ± 53.1
	100	..	10	106.3 ± 26.8
	200	..	10	208.5 ± 79.6
	..	100	10	36.6 ± 21.0
Chloral Hydrate	..	..	10	58.0 ± 22.8
400 mg./Kg., i.p.	50	..	10	101.3 ± 40.5
	100	..	10	221.9 ± 70.0
	200	..	5[c]	261.4 ± 56.7
	.	100	10	10.3 ± 6.2

[a] Number of survivors out of 10 mice injected.
[b] Mean sleeping time for 5 mice, the remaining 5 mice of this group did not lose the righting reflex.
[c] Number of survivors out of 10 mice injected.

muscle being elicited by stimuli applied every twenty-five seconds through a shielded electrode secured to the central end of the cut tibeal nerve. In six experiments responses of the opposite tibealis anterior to stimuli applied at twenty-five-second intervals to the peripheral end of the cut sciatic nerve were recorded concurrently with the flexion reflex. The stimulus used for eliciting the flexion reflex consisted of a tetanic induction shock of approximately 0.5 seconds duration from an inductorium; single break shocks were used for sciatic nerve stimulation. In the decerebrate and spinal cats at least two hours were allowed to elapse between removal of ether and beginning the record. The effect of T-Na was compared to that of mephenesin, a drug known to inhibit internuncial transmission. Both drugs were injected intravenously.

In the anesthetized cats, T-Na in doses of 30 and 40 mg /Kg. inhibited the flexion reflex to a degree comparable to that produced by similar doses of mephenesin (Fig. 1). In the decerebrate and spinal cats however, T-Na had an insignificant depressant action compared with that produced by mephenesin (Figs. 2 and 3). T-Na had no effect on the responses of the contralateral muscle to stimulation through its motor nerve.

Action on Frog Peripheral Nerve and Skeletal Muscle.—The nerve muscle preparation used here consisted of the iliofibularis muscle with its attached motor nerve and parent sciatic trunk, of which the method of isolation and use have been described by

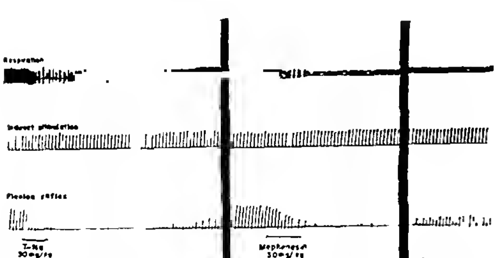

Fig. 1.—Anesthetized cat. Action of T-Na and mephenesin on tibealis anterior responses.

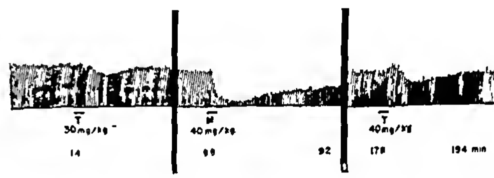

Fig. 2.—Decerebrate cat. Action of T-Na and mephenesin (M) on flexion reflex.

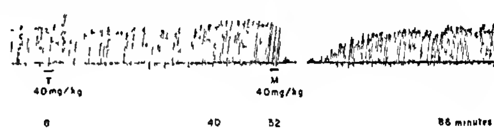

Fig. 3.—Spinal cat. Action of T-Na (T) and mephenesin (M) on flexion reflex.

Quilliam (20). Contractions were recorded with the preparation immersed in oxygenated frog Ringer's solution contained in a Palmer horizontal muscle chamber. When nerve and muscle were to be isolated, one from the other, the bath was divided by a specially constructed plexiglass partition. Stimuli consisted of single break shocks from an inductorium and were applied either directly to the muscle, to the motor nerve, or alternately to nerve and muscle at thirty-second intervals.

When T-Na was mixed into the bathing solution a gradual decline in the height of the muscle contractions occurred. If T-Na was left in the bath long enough the responses would decline eventually to zero or close to it. Recovery was slow, even after repeated washings, and seldom did the contractions regain the original height. Depression of responses occurred whether the muscle was stimulated directly or via its motor nerve, though responses to nerve stimulation were affected to a greater degree. When the nerve and muscle were in separate baths it was shown that the greater susceptibility of indirectly elicited responses was not due to depression of conduction in the motor nerve (Fig. 4).

Action on Blood Pressure and Heart Rate.—Blood pressure was recorded from the carotid artery in four cats and three dogs, anesthetized with pentobarbital sodium. Heart rate was determined

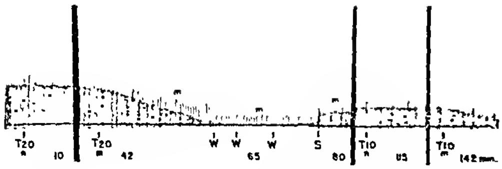

Fig. 4.—Isolated frog sciatic nerve-ilio-fibularis muscle preparation, nerve and muscle in separate chambers. Stimulus applied alternately to nerve or to muscle directly. This record shows the effect of T-Na 20 mg. % ($T20$) and of T-Na 10 mg. % ($T10$) when added to nerve chamber (n) or to muscle chamber (m). The m above the record indicates that the higher responses are those due to direct stimulation of the muscle.

from cardiograms recorded from lead II on an Edin electronic cardiograph, model 210. Intravenous injection of T-Na in doses of 25 to 50 mg./Kg. produced a temporary fall in blood pressure accompanied by a decrease in heart rate. The extent and rapidity of the fall in blood pressure, for a given dose, varied with the speed of injection. Both effects were more pronounced in cats than in dogs. Large doses (50 mg./Kg.), whether injected rapidly or slowly, caused complete paralysis of respiration. This effect was reversible and respiratory function returned after periods of fifteen to twenty minutes on artificial respiration (Fig. 5). The hypotensive effect remained unchanged after atropinization or bilateral section of the vagus. Responses to carotid occlusion and to injected epinephrine were not altered by an immediately previous injection of T-Na.

Action on Isolated Intestine.—Segments of ileum from rabbits and guinea pigs were suspended in a 50-cc. muscle chamber containing oxygenated Tyrode's solution and recordings made of their longitudinal contractions. The effect of T-Na was determined on spontaneous contractions and on the responses to the following intestinal stimulants: acetylcholine chloride, histamine acid phosphate, nicotine sulfate, barium chloride, and 5-hydroxytryptamine creatinine sulfate (serotonin). The final concentrations of the drugs in the muscle bath are expressed as mg. % of these salts.

In concentrations of 5 and 10 mg. % T-Na depressed spontaneous contractions of both rabbit and guinea-pig ileum with little or no effect on tone. On rabbit intestine, depression of spontaneous movements was much more pronounced when the contractions were of high magnitude. The responses to all of the stimulant drugs were inhibited, but not to the same extent, the responses to nicotine being inhibited most easily. On rabbit ileum this difference was such that T-Na appeared to be almost a selective antagonist of nicotine (Fig. 6). On guinea-pig ileum (Fig. 7) and when higher concentrations were used on rabbit ileum, a selective action against nicotine was less obvious.

DISCUSSION AND SUMMARY

The experimental evidence obtained in this investigation indicates that γ-thujaplicin sodium exerts a mixture of stimulant and depressant actions on the central nervous system of mammals. The stimulant component of its action is evident only in conscious animals and is manifested

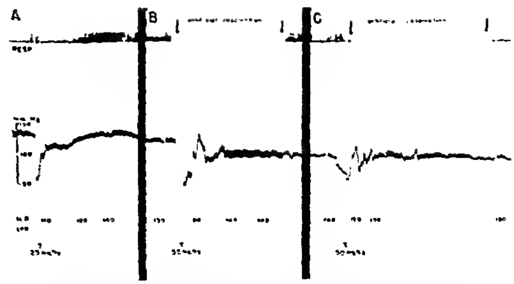

Fig. 5.—Anesthetized cat. Action of T-Na (T) on respiration, blood pressure, and heart rate ($H. R.$). In B and C artificial respiration was maintained until spontaneous respiratory movements were evident. Twenty minutes elapsed between A and B, sixteen minutes between B and C.

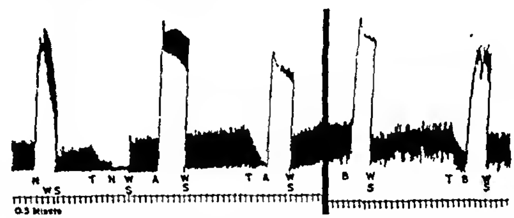

Fig. 6.—Isolated rabbit ileum. Effect of 10 mg. % T-Na (T) on responses to 150 mcg. % nicotine sulfate (N), 4 mcg. % acetylcholine (A), and 25 mg. % barium chloride (B). W is wash. Drum stopped at S.

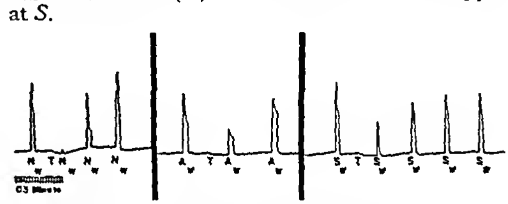

Fig. 7.—Guinea ileum. Effect of 5 mg. % T-Na (T) on responses to nicotine 500 mcg. % (N), acetylcholine 4 mcg. % (A), and serotonin 100 mcg. % (S). W is wash.

by convulsions which have been shown to be of cerebral origin. In this respect T-Na is similar to other volatile, cyclic ketones from natural sources, such as camphor and thujone. The depressant component of the action of T-Na appears to affect various levels of the mammalian central nervous system. The abolition of placing reactions observed in rats, rabbits, and cats indicates that cortical areas are depressed since a strictly localized cortical control of these reactions has been demonstrated in the cat by Bard (21) and in the rat by Brooks (22). In rodents, there was a suggestion that respiratory centers might be depressed by T-Na. Other depressant effects, which could be observed only when T-Na was given to previously narcotized animals, included the respiratory paralysis produced in pentobarbitalized cats and dogs, and the interneuronal blockade seen in anesthetized cats but not in spinal and decerebrate cats.

The results of the experiments in which T-Na was administered in conjunction with depressant drugs to mice show that T-Na is of no value as an analeptic. Toxic effects are involved in the alteration of sleeping times by the 200 mg./Kg. d
T-Na. The prolongation of thiopental and

hydrate sleeping times by T-Na in doses of 50 and 100 mg /Kg. may be further evidence of a central depressant action for T-Na However, since lower doses did not alter the sleeping times of all of the depressants used, it cannot be generally concluded that nontoxic doses of T-Na prolong sleeping times produced by depressant drugs

It has been reported that the central stimulant drugs picrotoxin and metrazol also possess some central depressant action (23), but compared with these useful analeptics, T-Na would appear to have a much higher ratio of depressant to stimulant activity

In frogs, the depressant action of T-Na is predominant and no signs of stimulation could be seen when the drug was injected intravenously or intralymphatically There may be a similarity here to the action of camphor, which produces a pronounced paralytic action on the spinal cord of frogs which entirely obscures other actions it may have higher up in the nervous system (24)

On other organs and systems which were studied, T-Na exhibited only depressant effects The temporary hypotension seen in anesthetized cats and dogs, and which was intensified when the drug was rapidly injected, seems most likely to be due to a direct depression of the myocardium resulting from its exposure to high concentrations of T-Na A central vagal action is ruled out by the persistence of the hypotensive response after atropinization and bilateral section of the vagi Interruption of sympathetic pathways does not seem likely since the responses to carotid occlusion and exogenous epinephrine were not altered by T-Na On the other hand, bradycardia following injection of T-Na paralleled the hypotensive response

Although there was no evidence that T-Na inhibited neuromuscular conduction in the cat anterior tibealis *in situ*, there was a suggestion of such an action on the isolated frog nerve-muscle preparation On this preparation, contractions due to stimulation of the motor nerve were reduced to a greater degree than those due to direct stimulation of the muscle This, together with the fact that T-Na did not inhibit conduction in the motor nerve, would appear to indicate a depressant effect at the neuromuscular junction This action, however, is not a potent one and develops slowly It may be that another point of similarity in the actions of T-Na and camphor is indicated here, since camphor has been reported to produce neuromuscular paralysis in frogs but not in mammals (25)

The depressant effect of T-Na on intestinal muscle obviously is not due to an atropine-like mechanism since atropine antagonizes exogenous acetylcholine more readily than it does nicotine, and furthermore, it does not prevent the stimulating effect of nicotine on rabbit intestine (26, 27, 28) On both guinea-pig and rabbit intestine, T-Na inhibited responses to nicotine more easily than those to other intestinal stimulants Since nicotine is the only one of the stimulant agents which, when applied exogenously, acts primarily to stimulate ganglia, it appears that T-Na, as well as depressing intestinal muscle, exerts also a depressant effect on myenteric ganglia Naess and Schanche attributed a similar type of action on guinea-pig intestine to barbiturates, which they found to inhibit nicotine responses more than those of other stimulant drugs (29). In view of the relatively high concentrations of T-Na required and the dissimilarity of structure between it and potent ganglionic-blocking agents such as hexamethonium, T-Na does not likely act as a competitive inhibitor at the ganglia Instead, this action is looked upon as another example of the general depressive action which has been exhibited on other tissues by T-Na.

Although no attempt was made in this investigation of γ-thujaplicin to duplicate the experiments by Lee (7, 8, 9) on β-thujaplicin, a limited comparison of central actions may be made Lee's reports indicate that on the central nervous system of mice, guinea pigs, and rabbits, β-thujaplicin, like T-Na, produces a combination of stimulant and depressant effects In the case of the beta isomer however, the depressant component of the central action appears to be more predominant For example, Lee's results show that subcutaneous doses of 500 mg /Kg of β-thujaplicin often caused death in guinea pigs without previous convulsions.

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Veratrum Alkaloids XXXV*

The Hypotensive Principles of Cryptenamine, A *Veratrum viride* Alkaloid Preparation

By S. MORRIS KUPCHAN and NORBERT GRUENFELD†

Cryptenamine has been reported to have a more favorable ratio of emetic to hypotensive dose than other veratrum preparations, and the advantageous ratio has been attributed to the presence of new hypotensive alkaloids. Cryptenamine was fractionated with the aid of partition column chromatography, adsorption column chromatography, and paper partition chromatography. The known hypotensive alkaloids protoveratrine A, protoveratrine B, germitrine, neogermitrine, germerine, and germidine were isolated. In addition, the relatively nonhypotensive alkaloids jervine, rubijervine, isorubijervine, and an apparently new noncarbonyl alkamine were obtained. Paper chromatographic evidence suggesting the presence of the hypotensive alkaloids germbudine and neogermbudine and the relatively nonhypotensive veratramine was also obtained.

Pure veratrum alkaloids and their preparations have been administered in recent years for the treatment of hypertension. The deterrent to the wider use of these agents in therapy is the narrow range between their therapeutic and emetic doses (1).

Cryptenamine, an alkaloidal preparation obtained from *Veratrum viride* by a nonaqueous benzene triethylamine extraction procedure has been reported to have a ratio of emetic to effective hypotensive dose superior to that of other veratrum preparations (2–5). In humans, the divergence between the hypotensive and the emetic doses was most apparent on intravenous administration (5). The satisfactory ambulatory treatment of arterial hypertension by oral administration of cryptenamine has also been reported (6). However, Abreu, *et al.* (7), reported in 1954 that cryptenamine did not demonstrate a ratio of emetic to hypotensive dose superior to that of protoveratrine A when tested in dogs.

The advantageous pharmacological properties of cryptenamine have been attributed to the presence of new amorphous hypotensive ester alkaloids reportedly lost by hydrolysis during the alternative aqueous ammonia-benzene extraction procedure (2–4). It was the purpose of this investigation to identify the components of cryptenamine which are responsible for its hypotensive activity.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11MS). Infrared spectra were determined in chloroform solution (unless otherwise specified) on a Baird double beam infrared recording spectrophotometer. Paper chromatography was conducted by the descending technique on Whatman No. 1 paper. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology and Huffman Microanalytical Laboratories, Wheatbridge, Colorado, on samples dried under reduced pressure.

Paper Chromatography.—The procedure and solvent systems employed were essentially those described by Fischbach and Levine (8, 9) for an ascending technique. This method involves the use of prewetted buffered paper and the detection of alkaloidal spots with a chloroform solution of bromphenol blue. Paper was impregnated with pH 3.5 McIlvaine buffer solution and dried. Before use this was rewetted with acetone-water (3:2) and samples (*ca.* 2 µl. of 3% solutions in chloroform) were applied. Approximately fifteen minutes after rewetting, the paper was placed into a tank equilibrated with respect to developing solvent and water. After a development period of four to five hours the solvent front had traveled about 15 inches. The paper was dried, sprayed with indicator, and exposed to a flow of steam. Cryptenamine alkaloids gave blue, blue green, or purple spots on a yellow background, the color given by each alkaloid helped in its identification by comparison with the behavior of known compounds.

The three solvent systems (8, 9) employed in this investigation were (*A*) *n*-butanol, *n*-butyl acetate, formic acid (5:25:1), (*B*) ethylene chloride, cellosolve acetate, pyridine (15:10:1), and (*C*) isooctane, methyl isobutyl ketone, pyridine (20:10:2). System *A* was employed to follow the course of most partition and adsorption chromatography column separations. Systems *A*, *B*, and *C* were used for comparison of cryptenamine components with authentic samples of known alkaloids.

Fractionation of Cryptenamine by Partition Chromatography.—Partition columns employing Celite 545 as support for the stationary phase consisting of a pH 4.25, 0.5 *M* citrate buffer were used for the frac-

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tionation of cryptenamine A slurry of Celite 545 (120 Gm) was made with benzene (1,000 cc) and the buffer solution (100 cc) was added in portions, the mixture was stirred vigorously after each addition The hydrated Celite was then packed into a column (3 cm diameter) in the usual manner and a solution of cryptenamine[1] (2 Gm , batch CaC64, in 3 cc of chloroform) was added to the column The column was eluted with appropriate solvents at a rate of *ca* 2 cc per minute and 100 cc fractions were collected The solvent was evaporated and the contents of each flask were weighed and submitted to paper chromatographic analysis using solvent system *A* The contents of fractions demonstrating a similar composition were combined and aliquots were submitted for pharmacological testing to ascertain hypotensive activities [2]

Variations were observed in the course of duplicate separations, probably due to differences in the degree of packing of columns, and thus the results obtained in two fractionations of the components of cryptenamine are summarized separately in Tables I and II The pharmacological data listed for the first column separation (Table I) indicates that the hypotensive principles of cryptenamine were concentrated in a few fractions The pharmacological data for the second separation demonstrated a similar distribution of hypotensive activity

Identification of Isolated Components.—Components were identified in all cases by comparison of infrared spectra and of paper chromatographic behavior in three solvent systems with those of authentic samples of known veratrum alkaloids In addition, the melting points were not depressed on admixture with authentic specimens of the characterized alkaloids Any additional confirmatory evidence is given individually for each component

Component I Isorubijervine [3]—Chloroform and acetone were added to fraction 1-A (Table I) Upon standing, needles [275 mg , m p 237–242° (decompn)] separated, $[\alpha]_D^{25}$ +8° (c 1 02, ethanol) This compound was identified as isorubijervine, and was also isolated (218 mg) from fraction 2 B (Table II) by the same crystallization procedure

Component II The New Alkamine—Addition of acetone to fraction 1-B (Table I) led to the separation of white velvety needles (85 mg) Recrystallization from chloroform methanol gave needles (45 mg), m p 258–260° (decompn), $[\alpha]_D^{25}$ +9° (c 1 1, pyr) This compound demonstrated only end absorption in the ultraviolet and no absorption in the carbonyl region of the infrared spectrum

Anal—Calcd for $C_{27}H_{43}NO_3$ C, 75 48, H, 10 09, N, 3 26 equiv wt , 429 6 Found C, 75 39, 75 83, H, 10 02, 10 38 N, 3 14, equiv wt , 434

The equivalent weight was determined by a nonaqueous titration method similar to that of Parks and Mitchner (11) Approximately 10 mg of alkaloid was dissolved in glacial acetic acid (10 cc) and 2 cc aliquots were titrated with standard 0 01 *N* $HClO_4$ in glacial acetic acid using *p* naphtholbenzein[4] (0 1% in glacial acetic acid) as indicator The solution was titrated to the appearance of a green color, the indicator changing from pink to green

This apparently new alkaloid, also isolated from fraction 2 E (Table II), demonstrated no hypotensive activity on the dog at an i v single dose of 4 mcg /Kg

Component III Neogermitrine—Fraction 1 C (Table I) in benzene was chromatographed on Merck acid-washed alumina (4 Gm , 0 7 cm diameter column) Elution with mixtures of benzene and chloroform (from benzene to chloroform benzene, 3 1) yielded a product (59 mg) shown to be predominantly component III on paper chromatographic analysis Crystallization from ether gave a compound [prisms, 20 mg , m p 235–237° (decompn)] identified as neogermitrine Neogermitrine [18 mg , m p 239–241° (decompn)] was obtained in a similar fashion by purification of fraction 2 H (Table II)

Component IV Protoveratrine A—Benzene solutions of fraction 2 F (50 mg) and fraction 2-E (47 mg remaining after removal of component II by crystallization) were combined and chromatographed on Merck acid washed alumina (2 5 Gm , 0 7 cm diameter column) Elution with chloroform benzene (1 1, 250 cc) yielded a product consisting mainly of component III (neogermitrine) Further elution with benzene-chloroform (from 65% chloroform to 100% chloroform) yielded a crude product (23 mg) consisting mainly of component IV Crystallization from ether led to isolation of protoveratrine A [prisms, 8 mg , m p 269–270° (decompn)]

Component V Jervine [3]—Cryptenamine (2 Gm in benzene) was chromatographed on acid washed alumina (50 Gm) The column was eluted with benzene absolute methanol (99 1, 700 cc), chloroform (300 cc), chloroform absolute methanol (99 1, 600 cc), chloroform absolute methanol (98 2, 350 cc), chloroform-absolute methanol (95 5, 600 cc), and chloroform absolute methanol (90 10, 300 cc) The eluates of the last two solvent combinations were combined on the basis of their paper chromatographic behaviors and evaporated to dryness Addition of acetone to the residue led to the crystallization of a compound [needles, 33 mg , m p 245–246° (decompn)], $[\alpha]_D^{25}$ −165° (c 1 17, ethanol), identified as jervine

Component VI Germitrine—The continued elution of the alumina column described for the isolation of component III (from fraction 1 C) with benzene chloroform (75% chloroform to pure chloroform) led to the isolation of a product (60 mg) consisting predominantly of component VI Crystallization from ether petroleum ether yielded germitrine [prisms, 10 mg , m p 220–222° (decompn)]

Germitrine was also isolated from fraction 2-J (Table II) by chromatography on acid washed alumina (3 5 Gm) Elution with benzene chloroform (3 1) removed colored nonalkaloidal material Further elution with benzene chloroform (1 1) yielded mainly component VI (32 mg) Crystallization from ether and recrystallization from same solvent gave germitrine [15 mg , m p 227–229° (decompn)]

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[1] We wish to thank Dr C J Cavallito of Irwin Neisler and Co for supplying us with the cryptenamine used for this investigation

[2] We wish to express our gratitude to Dr Thomas B O Dell of Irwin Neisler and Co for the pharmacological data reported in this paper Assays were performed by a procedure previously described (2)

[3] Dr Allan P Gray of Irwin Neisler and Co has kindly informed us of his unpublished independent isolation of isorubijervine jervine rubijervine and veratramine from cryptenamine

[4] We wish to thank Dr Kenneth A Connors for suggesting the use of this indicator

TABLE I.—FRACTIONATION OF CRYPTENAMINE[a] BY PARTITION CHROMATOGRAPHY
Experiment 1

Fraction	Eluent	Weight, mg	Hypotensive Units/mg[b]	Characterized Components— Alkaloids[c]	Estimated % of Total[d]
1-A	Benzene, 500 cc	450	Relatively inactive[e]	I	90
1-B[f]	Benzene, 400 cc	215	200	II, III, IV	90
1-C[f]	Benzene, 1,100 cc	220	900	III, V, VI, VII	90
1-D	Benzene, 800 cc	135	650	V, VI, VII, VIII	100
1-E	Benzene, 400 cc	75	Relatively inactive	V, VII, VIII	100
1-F	Benzene, 800 cc	165	Relatively inactive	V, VII, VIII	90
1-G	Benzene, 700 cc	90	Relatively inactive	V, VII, VIII	70
1-H	Benzene, 1,500 cc	130	250	IX	40
1-I	Benzene, 5,300 cc	175	200	IX, X	90
1-J	Chloroform, 800 cc	230	250	IX, X, XI	70
1-K	Chloroform-methanol, 98:2, 2,000 cc	140	Relatively inactive	XII, XIII	25
Total		2,025			80

[a] Batch CaC64, Irwin Neisler and Co., (2 Gm.), C.S.R. unit = 4.0 mcg./Kg.
[b] Number of hypotensive units/mg. = 1,000/C.S.R. unit (mcg./Kg.). One C.S.R. unit represents the amount of intravenously administered hypotensive agent per Kg. of body weight which just abolishes the pressor response to the carotid sinus reflex in dogs (10).
[c] I, isorubijervine, II, new alkamine, III, neogermitrine, IV, protoveratrine A, V, jervine, VI, germitrine, VII, veratramine, VIII, rubijervine, IX, germidine, X, germerine, XI, protoveratrine B, XII, germbudine, XIII, neogermbudine.
[d] The per cent of each fraction that the characterized components represent was estimated from the paper chromatogram of the fraction.
[e] No blood pressure fall observed with i.v. single dose of 4 mcg./Kg.
[f] Paper chromatographic analysis with solvent system *C* indicated the presence of a component not apparent with solvent system *A*. The presence of component IV was detected with solvent system *B*.

TABLE II.—FRACTIONATION OF CRYPTENAMINE[a] BY PARTITION CHROMATOGRAPHY
Experiment 2

Fraction	Eluent	Weight, mg.	Characterized Components— Alkaloids[b]	Estimated % of Total[c]
2-A	Benzene, 200 cc	96	I	50
2-B	Benzene, 200 cc	289	I	100
2-C	Benzene, 200 cc	70	I	75
2-D	Benzene, 200 cc	30	I, II	75
2-E	Benzene, 400 cc	171	II, III	85
2-F[d]	Benzene, 200 cc	50	II, III, IV	90
2-G[d]	Benzene, 200 cc	28	III, IV, V, VII	90
2-H	Benzene, 500 cc	94	III, V, VII	100
2-I	Benzene, 400 cc	59	III, V, VI, VII	100
2-J	Benzene, 800 cc	148	V, VI, VII	100
2-K	Benzene, 500 cc	91	V, VI, VII, VIII	100
2-L	Benzene, 1,100 cc	178	V, VII, VIII	100
2-M	Benzene, 700 cc	111	V, VII, VIII	90
2-N	Benzene, 400 cc	40	V	50
2-O	Benzene, 1,600 cc	61	None	0
2-P	Benzene, 1,400 cc	61	IX	40
2-Q	Benzene, 600 cc	31	IX	70
2-R	Benzene, 600 cc	21	IX	100
2-S	Benzene, 4,350 cc	37	IX, X	50
2-T	Benzene-chloroform, 3:1, 1,500 cc	76	X	80
2-U	Benzene-chloroform, 1:1, 1,500 cc	79	None	0
2-V	Benzene-chloroform, 1:1, 1,500 cc	51	XI	80
2-W	Benzene-chloroform, 1:3, 1,000 cc	31	XII, XIII	20
2-X	Chloroform, 1,500 cc	57	XII, XIII	30
2-Y	Chloroform-methanol, 98:2, 2,000 cc	55	XII, XIII	25
Total		2,015		77

[a] See footnote [a], Table I. [b] See footnote [c], Table I. [c] See footnote [d], Table I. [d] See footnote [f], Table I. In this fractionation, components IV and VI were clearly separated.

Component VIII: Rubijervine[3].—Fraction 1-E (Table I) was dissolved in hot chloroform and filtered. On standing, a crystalline compound separated (needles, 15 mg.), m. p. 247–248° (decompn.), $[\alpha]_D^{25}$ +9° (c 0.68, pyr.) identified as rubijervine. For the authentic sample of rubijervine, $[\alpha]_D^{25}$ = +7° (c 1.01, pyr.). The infrared spectra of the rubijervine samples were determined in KBr pellets.

Component IX: Germidine.—A benzene solution of fraction 1-I (Table I) was chromatographed on Merck acid-washed alumina (4 Gm., 0.7 cm. diameter column). Elution with benzene-chloroform (75% chloroform to pure chloroform) yielded a crude product (25 mg.) consisting mainly of component IX. Crystallization from ether gave germidine [prisms, 5 mg., m. p. 235–237° (decompn.)].

Component X: Germerine.—Continued elution of the column described for the isolation of component IX with chloroform and chloroform-methanol

tionation of cryptenamine A slurry of Celite 545 (120 Gm) was made with benzene (1,000 cc) and the buffer solution (100 cc) was added in portions, the mixture was stirred vigorously after each addition The hydrated Celite was then packed into a column (3 cm diameter) in the usual manner and a solution of cryptenamine[1] (2 Gm, batch CaC64, in 3 cc of chloroform) was added to the column The column was eluted with appropriate solvents at a rate of *ca* 2 cc per minute and 100 cc fractions were collected The solvent was evaporated and the contents of each flask were weighed and submitted to paper chromatographic analysis using solvent system *A* The contents of fractions demonstrating a similar composition were combined and aliquots were submitted for pharmacological testing to ascertain hypotensive activities [2]

Variations were observed in the course of duplicate separations, probably due to differences in the degree of packing of columns, and thus the results obtained in two fractionations of the components of cryptenamine are summarized separately in Tables I and II The pharmacological data listed for the first column separation (Table I) indicates that the hypotensive principles of cryptenamine were concentrated in a few fractions The pharmacological data for the second separation demonstrated a similar distribution of hypotensive activity

Identification of Isolated Components.—Components were identified in all cases by comparison of infrared spectra and of paper chromatographic behavior in three solvent systems with those of authentic samples of known veratrum alkaloids In addition, the melting points were not depressed on admixture with authentic specimens of the characterized alkaloids Any additional confirmatory evidence is given individually for each component

Component I Isorubijervine [3]—Chloroform and acetone were added to fraction 1-A (Table I) Upon standing, needles [275 mg, m p 237–242° (decompn)] separated, $[\alpha]^{25}_{D}$ +8° (c 1 02, ethanol) This compound was identified as isorubijervine, and was also isolated (218 mg) from fraction 2-B (Table II) by the same crystallization procedure

Component II The New Alkamine—Addition of acetone to fraction 1 B (Table I) led to the separation of white velvety needles (85 mg) Recrystallization from chloroform methanol gave needles (45 mg), m p 258–260° (decompn), $[\alpha]^{25}_{D}$ +9° (c 1 1, pyr) This compound demonstrated only end absorption in the ultraviolet and no absorption in the carbonyl region of the infrared spectrum

Anal—Calcd for $C_{?}H_{43}NO_3$ C, 75 48, H, 10 09, N, 3 26 equiv wt, 429 6 Found C, 75 39, 75 83, H, 10 02, 10 38 N, 3 14, equiv wt, 434

The equivalent weight was determined by a nonaqueous titration method similar to that of Parks and Mitchner (11) Approximately 10 mg of alkaloid was dissolved in glacial acetic acid (10 cc) and 2 cc aliquots were titrated with standard 0 01 *N* $HClO_4$ in glacial acetic acid using *p* naphthol benzein[4] (0 1% in glacial acetic acid) as indicator The solution was titrated to the appearance of a green color, the indicator changing from pink to green

This apparently new alkaloid, also isolated from fraction 2 E (Table II), demonstrated no hypotensive activity on the dog at an i v single dose of 4 mcg /Kg

Component III Neogermitrine—Fraction 1 C (Table I) in benzene was chromatographed on Merck acid-washed alumina (4 Gm, 0 7-cm diameter column) Elution with mixtures of benzene and chloroform (from benzene to chloroform benzene, 3 1) yielded a product (59 mg) shown to be predominantly component III on paper chromatographic analysis Crystallization from ether gave a compound [prisms, 20 mg, m p 235–237° (decompn)] identified as neogermitrine Neogermitrine [18 mg, m p 239–241° (decompn)] was obtained in a similar fashion by purification of fraction 2 H (Table II)

Component IV Protoveratrine A—Benzene solutions of fraction 2-F (50 mg) and fraction 2-E (47 mg remaining after removal of component II by crystallization) were combined and chromatographed on Merck acid-washed alumina (2 5 Gm, 0 7 cm diameter column) Elution with chloroform-benzene (1 1, 250 cc) yielded a product consisting mainly of component III (neogermitrine) Further elution with benzene-chloroform (from 65% chloroform to 100% chloroform) yielded a crude product (23 mg) consisting mainly of component IV Crystallization from ether led to isolation of protoveratrine A [prisms, 8 mg, m p 269–270° (decompn)]

Component V Jervine [3]—Cryptenamine (2 Gm in benzene) was chromatographed on acid washed alumina (50 Gm) The column was eluted with benzene absolute methanol (99 1, 700 cc), chloroform (300 cc), chloroform absolute methanol (99 1, 600 cc), chloroform absolute methanol (98 2, 350 cc), chloroform absolute methanol (95 5, 600 cc), and chloroform absolute methanol (90 10, 300 cc) The eluates of the last two solvent combinations were combined on the basis of their paper chromatographic behaviors and evaporated to dryness Addition of acetone to the residue led to the crystallization of a compound [needles, 33 mg, m p 245–246° (decompn)], $[\alpha]^{25}_{D}$ −165° (c 1 17, ethanol), identified as jervine

Component VI Germitrine—The continued elution of the alumina column described for the isolation of component III (from fraction 1-C) with benzene chloroform (75% chloroform to pure chloroform) led to the isolation of a product (60 mg) consisting predominantly of component VI Crystallization from ether petroleum ether yielded germitrine [prisms, 10 mg, m p 220–222° (decompn)]

Germitrine was also isolated from fraction 2-J (Table II) by chromatography on acid washed alumina (3 5 Gm) Elution with benzene chloroform (3 1) removed colored nonalkaloidal material Further elution with benzene chloroform (1 1) yielded mainly component VI (32 mg) Crystallization from ether and recrystallization from same solvent gave germitrine [15 mg, m p 227–229° (decompn)]

[1] We wish to thank Dr C J Cavallito of Irwin Neisler and Co for supplying us with the cryptenamine used for this investigation

[2] We wish to express our gratitude to Dr Thomas B O Dell of Irwin Neisler and Co for the pharmacological data reported in this paper Assays were performed by a procedure previously described (2)

[3] Dr Allan P Gray of Irwin Neisler and Co has kindly informed us of his unpublished independent isolation of isorubijervine jervine rubijervine and veratramine from cryptenamine

[4] We wish to thank Dr Kenneth A Connors for suggesting the use of this indicator

TABLE I.—FRACTIONATION OF CRYPTENAMINE[a] BY PARTITION CHROMATOGRAPHY
Experiment 1

Fraction	Eluent	Weight, mg	Hypotensive Units/mg [b]	Characterized Components— Alkaloids[c]	Estimated % of Total[d]
1-A	Benzene, 500 cc	450	Relatively inactive[e]	I	90
1-B[f]	Benzene, 400 cc	215	200	II, III, IV	90
1-C[f]	Benzene, 1,100 cc	220	900	III, V, VI, VII	90
1-D	Benzene, 800 cc	135	650	V, VI, VII, VIII	100
1-E	Benzene, 400 cc	75	Relatively inactive	V, VII, VIII	100
1-F	Benzene, 800 cc	165	Relatively inactive	V, VII, VIII	90
1-G	Benzene, 700 cc	90	Relatively inactive	V, VII, VIII	70
1-H	Benzene, 1,500 cc	130	250	IX	40
1-I	Benzene, 5,300 cc	175	200	IX, X	90
1-J	Chloroform, 800 cc	230	250	IX, X, XI	70
1-K	Chloroform-methanol, 98:2, 2,000 cc.	140	Relatively inactive	XII, XIII	25
Total		2,025			80

[a] Batch CaC64, Irwin, Neisler and Co., (2 Gm.), C.S.R. unit = 4.0 mcg./Kg.
[b] Number of hypotensive units/mg. = 1,000/C.S.R. unit (mcg./Kg.). One C.S.R. unit represents the amount of intravenously administered hypotensive agent per Kg. of body weight which just abolishes the pressor response to the carotid sinus reflex in dogs (10).
[c] I, isorubijervine, II, new alkamine, III, neogermitrine, IV, protoveratrine A, V, jervine, VI, germitrine, VII, veratramine, VIII, rubijervine, IX, germidine, X, germerine, XI, protoveratrine B, XII, germbudine, XIII, neogermbudine.
[d] The per cent of each fraction that the characterized components represent was estimated from the paper chromatogram of the fraction.
[e] No blood pressure fall observed with i. v. single dose of 4 mcg./Kg.
[f] Paper chromatographic analysis with solvent system *C* indicated the presence of a component not apparent with solvent system *A*. The presence of component IV was detected with solvent system *B*.

TABLE II.—FRACTIONATION OF CRYPTENAMINE[a] BY PARTITION CHROMATOGRAPHY
Experiment 2

Fraction	Eluent	Weight, mg	Characterized Components— Alkaloids[b]	Estimated % of Total[c]
2-A	Benzene, 200 cc	96	I	50
2-B	Benzene, 200 cc	289	I	100
2-C	Benzene, 200 cc	70	I	75
2-D	Benzene, 200 cc	30	I, II	75
2-E	Benzene, 400 cc	171	II, III	85
2-F[d]	Benzene, 200 cc	50	II, III, IV	90
2-G[d]	Benzene, 200 cc	28	III, IV, V, VII	90
2-H	Benzene, 500 cc	94	III, V, VII	100
2-I	Benzene, 400 cc	59	III, V, VI, VII	100
2-J	Benzene, 800 cc	148	V, VI, VII	100
2-K	Benzene, 500 cc	91	V, VI, VII, VIII	100
2-L	Benzene, 1,100 cc	178	V, VII, VIII	100
2-M	Benzene, 700 cc	111	V, VII, VIII	90
2-N	Benzene, 400 cc	40	V	50
2-O	Benzene, 1,600 cc	61	None	0
2-P	Benzene, 1,400 cc	61	IX	40
2-Q	Benzene, 600 cc	31	IX	70
2-R	Benzene 600 cc	21	IX	100
2-S	Benzene, 4,350 cc	37	IX, X	50
2-T	Benzene-chloroform, 3:1, 1,500 cc	76	X	80
2-U	Benzene-chloroform, 1:1, 1,500 cc	79	None	0
2-V	Benzene-chloroform, 1:1, 1,500 cc	51	XI	80
2-W	Benzene-chloroform, 1:3, 1,000 cc	31	XII, XIII	20
2-X	Chloroform, 1,500 cc	57	XII, XIII	30
2-Y	Chloroform-methanol, 98:2, 2,000 cc	55	XII, XIII	25
Total		2,015		77

[a] See footnote [a], Table I. [b] See footnote [c], Table I. [c] See footnote [d], Table I. [d] See footnote [f], Table I. In this fractionation, components IV and VI were clearly separated.

Component VIII: Rubijervine[3].—Fraction 1-E (Table I) was dissolved in hot chloroform and filtered. On standing, a crystalline compound separated (needles, 15 mg.), m. p. 247–248° (decompn.), $[\alpha]^{25}_{D}$ +9° (c 0.68, pyr.) identified as rubijervine. For the authentic sample of rubijervine, $[\alpha]^{25}_{D} = +7°$ (c 1.01, pyr.). The infrared spectra of the rubijervine samples were determined in KBr pellets.

Component IX: Germidine.—A benzene solution of fraction 1-I (Table I) was chromatographed on Merck acid-washed alumina (4 Gm., 0.7-cm. diameter column). Elution with benzene-chloroform (75% chloroform to pure chloroform) yielded a crude product (25 mg.) consisting mainly of component IX. Crystallization from ether gave germidine [prisms, 5 mg., m. p. 235–237° (decompn.)].

Component X: Germerine.—Continued elution of the column described for the isolation of component IX with chloroform and chloroform-methanol

(from 0.5 to 5% methanol) yielded a crude product (40 mg.) consisting mainly of component X. Crystallization from ether-petroleum ether led to the isolation of germerine [prisms, 6 mg., m. p. 201.5–203.5° (decompn.)].

Germerine was also isolated from fraction 2-T (Table II). A benzene solution of fraction 2-T was chromatographed on Merck acid-washed alumina (1 Gm., 0.7-cm. diameter column). Elution with benzene-chloroform (1:1, 100 cc.) gave nonalkaloidal colored material. Further elution with chloroform-methanol (95:5, 100 cc.), evaporation of eluate to dryness and crystallization from ether-petroleum ether yielded germerine [prisms, 17 mg., m. p. 196–198° (decompn.)].

Component XI: Protoveratrine B.—A benzene solution of fraction 2-V (Table II) was chromatographed on Merck acid-washed alumina (1 Gm., 0.7-cm. diameter column). Elution with benzene led to the removal of colored material. Further elution with chloroform-methanol (98:2), evaporation of eluate to dryness and crystallization from ether led to the isolation of 4 mg. of protoveratrine B.

Evidence for Identification of Other Components.—*Component VII: Veratramine.*[3]—Paper chromatographic analysis of cryptenamine and of certain fractions thereof indicated the presence of a component with R_f identical with that of veratramine. The best separation of component VII was achieved with solvent system *A*.

Component XII: Germbudine.—Paper chromatographic analysis of fractions 1-K (Table I) and 2-W, 2-X, 2-Y (Table II) suggested the presence of a compound having an R_f identical with that of germbudine; it was best separated from other components with solvent system *B* (development period, twenty hours).

Component XIII: Neogermbudine.—Paper chromatographic analysis of fractions 1-K (Table I) and 2-W, 2-X, 2-Y (Table II) suggested the presence of a compound having an R_f identical with that of neogermbudine. Component XIII could also be best separated from other components with solvent system *B* (development period, twenty hours). Neogermbudine demonstrated a higher R_f than germbudine.

RESULTS AND DISCUSSION

As described above, cryptenamine was fractionated by partition and adsorption column chromatography; the progress of separations was followed by paper chromatographic analysis. By this procedure, during which cryptenamine was never subjected to conditions favorable for ester hydrolysis, the known (12, 13) hypotensive alkaloids protoveratrine A (IV), protoveratrine B (XI), neogermitrine (III), germitrine (VI), germidine (IX), and germerine (X) were isolated. In addition, it was possible to isolate the relatively nonhypotensive alkaloids (14) isorubijervine (I), jervine (V), rubijervine (VIII), and an apparently new noncarbonyl alkamine (II). Paper chromatographic evidence suggesting the presence of hypotensively active germbudine (XII) (15) and neogermbudine (XIII) (16) and of relatively inactive veratramine (VII) (14) was also obtained. It was estimated that the components listed represent some 80%, by weight, of cryptenamine (Tables I and II).

TABLE III.—HYPOTENSIVE PRINCIPLES OF ACTIVE FRACTIONS[a]

Fraction	Estimated % Characterized[b]	Hypotensive Alkaloids Identified	Total Hypotensive Units[c]
1-B	90	Protoveratrine A, neogermitrine	43,000
1-C	90	Neogermitrine, germitrine	198,000
1-D	100	Germitrine	88,000
1-H	40	Germidine	32,000
1-I	90	Germidine, germerine	35,000
1-J	70	Germidine, germerine, protoveratrine B	58,000
Total	80	...	454,000
Cryptenamine	80	...	500,000

[a] Fractions from partition chromatography (Table I) of 2 Gm. of cryptenamine.
[b] See footnote[d], Table I.
[c] Obtained by multiplication of weight of fraction by hypotensive units/mg. Cryptenamine assay, 250 hypotensive units/mg.

The pharmacological results summarized in Table III indicate that the six highly active fractions obtained on chromatographic separation of cryptenamine (Table I) represent approximately 90% of the total hypotensive activity of cryptenamine. The estimated proportion of total characterized components in these fractions is 80% and the active principles identified in these fractions represent the most powerful hypotensive alkaloids of *Veratrum viride* (7, 17). In view of the foregoing, it appears likely that known ester alkaloids are responsible for most of the hypotensive activity of cryptenamine.

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Veratrum Alkaloids XXXVII*

The Structure of Germanitrine, A Hypotensive Ester Alkaloid

By S. MORRIS KUPCHAN and ADRIANO AFONSO

The structure of germanitrine, a highly active hypotensive ester alkaloid, has been elucidated. Germine 3-angelate 7-acetate 15-*l*-2′-methylbutyrate, synthesized from germine, was found to be identical with germanitrine.

GERMANITRINE is a highly active hypotensive ester alkaloid isolated from *Veratrum fimbriatum* Gray (1). Alkaline hydrolysis of germanitrine has afforded the known alkamine germine (I) (2) and one mole equivalent each of acetic, tiglic, and *l*-2-methylbutyric acids (1). Methanolysis of germanitrine resulted in loss of the acetate grouping with conversion to the diester germanidine (1). The latter compound yielded one mole equivalent of tiglic acid and one mole equivalent of *l*-2-methylbutyric acid on alkaline hydrolysis. The positions of the esterifying acids on the germine nucleus have now been established, thereby effecting the elucidation of the complete structures of germanitrine and germanidine.

Tentative assignment of the acetate group of germanitrine to C_7 of the germine nucleus has been made on the basis of its reactivity toward methanolysis and its characteristically high contribution to the molecular rotation of the triester (3). A common feature of the naturally-occurring germine triesters of known structure is attachment of the three acyl moieties to positions C_3, C_7, and C_{15} (3, 4), and it appeared reasonable as a preliminary working hypothesis to assign the tiglate and *l*-2-methylbutyrate residues to C_3 and C_{15}. The similarity of the optical rotations of germanidine (1) to those of all other known C_3, C_{15} diesters of germine (3) supported a 3,15-diester structure for germanidine. Because only small comparison samples of germanitrine and germanidine were available to us, a degradative structure elucidation was precluded. Instead, a partial synthesis of germanidine and germanitrine starting with germine was undertaken.

The first objective selected was germine 3-tiglate 15-*l*-2′-methylbutyrate. Germine 15-*l*-2′-methylbutyrate (II) (4, 5) was treated with a limited amount of tigloyl chloride in pyridine to yield the desired diester III. The tiglate group of III was assigned to C_3 on the following bases. The diester was stable to periodic acid, an indication that no free 1,2-glycol system was present. This fact limited the possible sites of attachment of the tiglate residue to C_3 or C_4. The diester consumed two oxygen equivalents of chromic acid, which indicated that only two secondary hydroxyl groups were present and that the tiglate residue was therefore affixed at C_3. The infrared spectrum of germine 3-tiglate 15-*l*-2′-methylbutyrate (III) differed significantly from that of germanidine.

The next alternative explored was the hypothetical formulation of germanidine as a 3-*l*-2′-methylbutyrate 15-tiglate. Treatment of germine with a limited amount of tigloyl chloride in pyridine afforded germine 15-tiglate (IV). That the tiglate was affixed to C_{15} was shown by the periodic acid consumption of one mole equivalent. Only a C_{15} monoester of germine possesses a single 1,2-glycol system. Furthermore, the amorphous periodic acid cleavage product showed infrared absorption at 3.65, 5 62, and 5.80 μ characteristic of the aldehydo-γ-lactone resulting from cleavage of the ring A glycol (2). Germine 15-tiglate (IV) was treated with a limited amount of *l*-2-methylbutyryl chloride in pyridine to yield germine 3-*l*-2′-methylbutyrate 15-tiglate (V). Evidence for the 3,15-diester formulation V paralleled that for III. The infrared spectrum of V also differed significantly from that of germanidine.

The original proposal that the tiglate, rather than the geometrically isomeric angelate residue was present in germanitrine was based on the identification of *p*-phenylphenacyl tiglate among the *p*-phenylphenacyl esters derived from the acids formed upon hydrolysis (1). As a control, it was shown that neither pure angelic nor pure tiglic acid isomerized appreciably under the alkaline conditions employed for the hydrolysis of the ester alkaloids (1). On the other hand, it appeared reasonable that isomerization of angelate to tiglate might have occurred prior to or during hydrolysis of the ester alkaloids, or even during the preparation of the *p*-phenylphenacyl esters.

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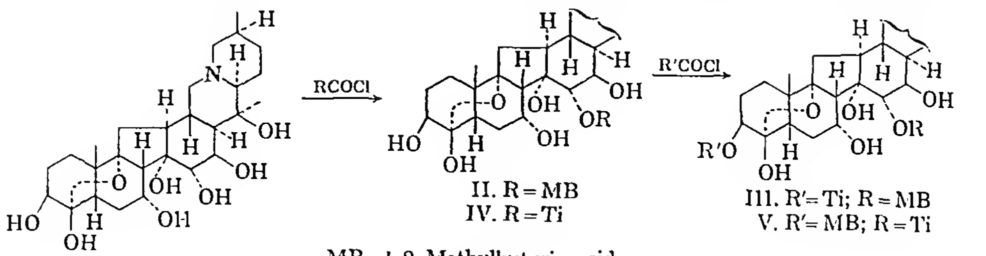
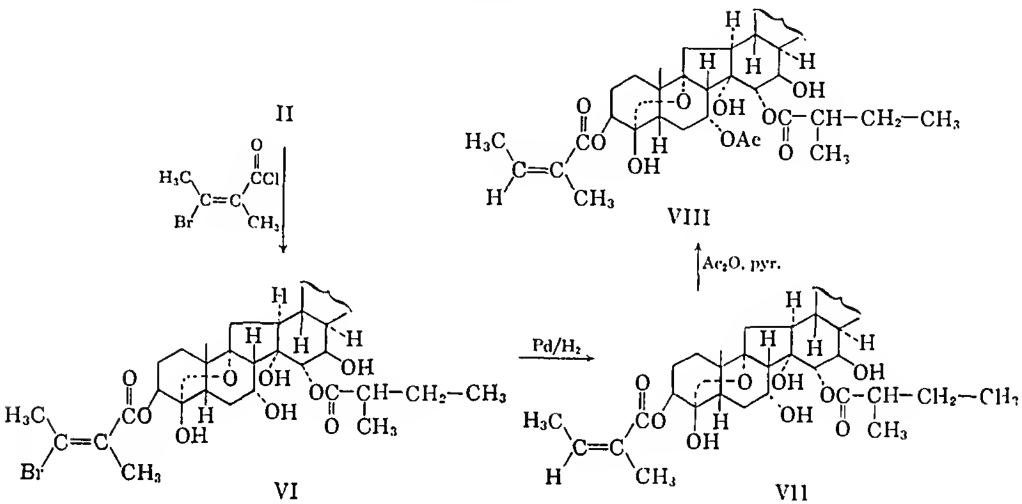


MB = *l*-2-Methylbutyric acid
Ti = Tiglic acid



The historical differences concerning the acid residue present in the schoenocaulon ester alkaloid cevadine are best rationalized on the basis that isomerization of the angelate residue to tiglate may take place prior to or during hydrolysis. In the case of cevadine, some authors reported hydrolysis to tiglic acid (6, 7), some to angelic acid (8, 9, 10), and some to a mixture of angelic and tiglic acids (11).

A partial synthesis of germine 3-angelate 15-*l*-2′-methylbutyrate (VII) was next undertaken. Germine 15-*l*-2′-methylbutyrate (II) was treated with a limited amount of 3-bromoangeloyl chloride (12) to yield germine 3-(3′-bromoangelate) 15-*l*-2′-methylbutyrate (VI). The latter compound, on hydrogenolysis, afforded germine 3-angelate 15-*l*-2′-methylbutyrate (VII). Evidence for the 3,15-diester formulation VII paralleled that for III. The melting point of the synthetic diester was not depressed on admixture with an authentic sample of germanidine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

Acetylation of germanidine (VII) with a limited amount of acetic anhydride yielded the triester germine 3-angelate 7-acetate 15-*l*-2′-methylbutyrate (VIII). Evidence for the attachment of the acetate group at C_7 was obtained in the following manner. A chromic acid titration of the triester showed the presence of only one secondary hydroxyl group and C_4 was thereby excluded as a site for attachment of the acetate ester. Furthermore, the oxidation product, on alkaline treatment, afforded a diosphenol with ultraviolet spectral properties identical with those of the diosphenol from neogermitrone and 16-dehydrogermine 3,4,7,15-tetraacetate (3). The triester showed no depression in melting point on admixture with an authentic sample of germanitrine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Infrared spectra were determined in chloroform on a Baird double beam recording spectrophotometer. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology on samples dried under reduced pressure at 110°.

Periodic Acid Titrations.—These titrations were

performed as in Part XXVIII (2) except that one-third of the total volume of water was replaced with *t*-butanol.

Chromic Acid Titrations.—The method described in Part XXXII (4) was used. Results are summarized in Table I.

Paper Chromatography. The technique followed was that described by Levine and Fischbach (13). Unless otherwise specified the *n*-butyl acetate: *n*-butanol: formic acid (25:5:1 by volume) system was used.

Germine 3-Tiglate 15-l-2'-Methylbutyrate (III).—To germine 15-*l*-2'-methylbutyrate (5) (0.4 Gm.) in pyridine (8 cc.) cooled to 0°, was added tigloyl chloride (14) (0.9 Gm.) with stirring. The solution was kept at room temperature for twenty-four hours. The reaction mixture was then cooled by adding crushed ice, made alkaline (pH 8) with a 10% sodium carbonate solution, and extracted several times with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in benzene and the solution was evaporated to dryness. This procedure was repeated until all traces of pyridine had been removed. The amorphous residue was chromatographed on Merck acid-washed alumina (10 Gm.), and the progress of the column separation was followed with a paper chromatogram of each fraction collected. Elution with benzene (100 cc.) yielded a substance (90 mg.) having a high R_f, presumably a triester. Germine 3-tiglate 15-*l*-2'-methylbutyrate (III, 0.275 Gm.) was eluted with 50% benzene-chloroform (400 cc.). The diester III was crystallized from acetone-ether as pale yellow prisms (150 mg.), m. p. 227–228° (decompn.); $[\alpha]^{21}_{D} + 3°$ (c 2.65, pyr.).

Anal.—Calcd. for $C_{37}H_{57}NO_{10}$: C, 65.77; H, 8.44. Found: C, 65.39; H, 8.58.

In a volatile acid determination (15) 15.31 mg. of the compound yielded an amount of acid equivalent to 5.05 cc. of 0.009181 *N* sodium thiosulfate; calcd. for 1 mole equivalent of tiglic acid and 1 mole equivalent of *l*-2-methylbutyric acid as required by structure III, 4.94 cc.

Diester III did not consume any periodic acid after six and one-half hours. The infrared spectrum of III was not identical with that of germanidine.

Germine 15-Tiglate (IV).—To a cold (0°) solution of germine (3.2 Gm.) in pyridine (25 cc.) was added tigloyl chloride (14) (0.9 Gm.). The mixture was stirred until homogeneous, allowed to warm to room temperature and stand for twenty-four hours. Further treatment followed the procedure used for III. The amorphous reaction product (3.27 Gm.) was chromatographed on Merck acid-washed alumina (60 Gm.). The separation was followed with a paper chromatogram of each fraction collected, using the resolving system for slow-moving alkaloids (13)[1] Elution with chloroform (1,000 cc.) yielded a product having a high R_f, presumably a diester. Further elution with 3% methanol-chloroform afforded germine 15-tiglate (IV) (1.16 Gm.) which was crystallized from chloroform-acetone as colorless prisms (720 mg.), m. p. 224–225° (decompn.), $[\alpha]^{21}_{D} - 7°$ (c 2.15, pyr.).

[1] The solution was prepared by adding 1 cc. of formic acid to the separated organic layer of the system *n*-butyl acetate: *n*-butanol. water (10:25:10 by volume).

TABLE I.—CHROMIC ACID TITRATIONS

Ester	Oxygen Equivalents Consumed 1 hr.	4 hr.
III	1.8	2.3
V	1.6	1.9
VII	1.7	2.1
VIII	1.3	1.4

Anal.—Calcd. for $C_{32}H_{49}NO_9$: C, 64.95; H, 8.35. Found: C, 65.22; H, 8.24.

In a volatile acid determination (15) 15.46 mg. of the compound yielded an amount of acid equivalent to 2.00 cc. of 0.009181 *N* sodium thiosulfate; calcd. for 1 mole equivalent of tiglic acid as required for structure IV, 2.80 cc.

Monoester IV consumed 1.1 mole equivalents of periodic acid after one hour and was stable thereafter. The solution remaining from the periodic acid oxidation was made alkaline (pH 8) with dilute ammonia and extracted with chloroform. The chloroform extracts were dried over sodium sulfate and the amorphous residue left on evaporation showed infrared absorption at 3.65, 5.62, and 5.80 μ.

Germine 3-l-2'-Methylbutyrate 15-Tiglate (V).—Germine 15-tiglate (IV) (0.4 Gm.) in pyridine (4 cc.) was treated with *l*-2-methylbutyryl chloride (5) (0.116 Gm.) under the same conditions used for diester III. The amorphous reaction product was chromatographed on Merck acid-washed alumina (10 Gm.). Elution with benzene (100 cc.) yielded a product (0.12 Gm.) having a high R_f, presumably a triester. Germine 3-*l*-2'-methylbutyrate 15-tiglate (V) (0.2 Gm.) was eluted with 50% benzene-chloroform (540 cc.) and crystallized with difficulty from acetone, as colorless plates (55 mg.), m. p. 180–181° (decompn.); $[\alpha]^{21}_{D} + 3°$ (c 1.10, pyr.).

Anal.—Calcd. for $C_{37}H_{57}NO_{10}$: C, 65.77; H, 8.44. Found: C, 65.35; H, 8.70.

In a volatile acid determination (15) 8.0 mg. of the compound yielded an amount of acid equivalent to 2.70 cc. of 0.009181 *N* sodium thiosulfate; calcd. for 1 mole equivalent of tiglic acid and 1 mole equivalent of *l*-2-methylbutyric acid as required by structure V, 2.58 cc.

Diester V was stable toward periodic acid. The infrared spectrum of V was not identical with that of germanidine.

Germine 3-Angelate 15-l-2'-Methylbutyrate (VII).—To a solution of germine 15-*l*-2'-methylbutyrate (0.365 Gm.) in pyridine (5 cc.) was added with stirring, 3-bromoangeloyl chloride (12) (0.135 Gm.) at room temperature. The reaction mixture was allowed to stand for twenty-four hours and further treatment followed the procedure used for diester III. The reaction products from two such batches were combined (0.92 Gm.) and chromatographed on Merck acid-washed alumina (16 Gm.). Elution with benzene (250 cc.) yielded an amorphous product (0.07 Gm.) with a high R_f, presumably a triester. Elution with 50% benzene-chloroform (500 cc.) followed by chloroform (870 cc.) yielded germine 3-(3'-bromoangelate) 15-*l*-2'-methylbutyrate (VI) (0.312 Gm.) as a pale yellow amorphous material, $[\alpha]^{21}_{D} + 16°$ (c 1.05, ethanol).

To a suspension of 10% palladium on carbon (0.07 Gm.) in 95% ethanol (10 cc.) which had previously been saturated with hydrogen, was added a solution

of VI (0.14 Gm.) in ethanol (4 cc.) containing anhydrous sodium acetate (0.07 Gm.). After hydrogenation at atmospheric pressure for three minutes the theoretical uptake of one mole equivalent of hydrogen was complete and the hydrogenation was terminated. The catalyst was removed by filtration, washed with ethanol (15 cc.), and the filtrate was evaporated under reduced pressure. The residue was suspended in water (5 cc.), made alkaline (pH 8) with a 10% sodium carbonate solution, and extracted several times with chloroform. The chloroform extract was dried over anhydrous sodium sulfate. Evaporation under reduced pressure yielded germine 3-angelate 15-*l*-2′-methylbutyrate (0.130 Gm.) which was crystallized from acetone-water as colorless flat needles (115 mg.), m. p. 220–221° (decompn.), $[\alpha]^{21}_{D}$ −3° (c 1.20, pyr.). The melting point was not depressed on admixture with an authentic sample of germanidine.[2]

Diester VII was stable to periodic acid. The infrared spectra and paper chromatographic behavior of VII and an authentic sample of germanidine[2] were identical.

Germine 3-Angelate 7-Acetate 15-l-2′-Methylbutyrate (VIII).—To a solution of VII (0.120 Gm.) in pyridine (1 cc.) was added acetic anhydride (0.021 Gm.) at 0°. The reaction mixture was allowed to stand at room temperature for twelve hours. Excess acetic anhydride was destroyed with two drops of methanol and the mixture was evaporated under reduced pressure at room temperature. The residue was dissolved in benzene and evaporated to dryness. This procedure was repeated several times to remove traces of pyridine. The residue was dissolved in ice water (3 cc.), made alkaline (pH 8) with 10% sodium carbonate solution, extracted with chloroform, and the extract evaporated to dryness. The amorphous residue (0.120 Gm.) was dissolved in chloroform and petroleum ether (Skelly B) was added to the solution until an amorphous precipitate separated. The precipitate was removed by filtration and rejected. The filtrate on concentration deposited crystalline triester VIII (0.055 Gm.) which had the same R_f as germanitrine.[2] Recrystallization from acetone-water afforded colorless needles (50 mg.), m. p. 225–226° (decompn.). The melting point was not depressed on admixture with an authentic sample of germanitrine.[2] The infrared spectrum of triester VIII was identical with that of germanitrine.

Chromic Acid Oxidation of Germanitrine.—The chromic acid titrations of germanitrine (Table I) were performed on two 2-cc. aliquot parts taken from a solution (10 cc.) containing 29.1 mg. of germanitrine. The solution remaining after removal of the aliquots was treated with a sodium bisulfite solution until green, made alkaline with 20% NaOH, and extracted with chloroform. The extract was evaporated to dryness under reduced pressure. The residue was dissolved in 1% methanolic sodium hydroxide (4 cc.) and heated under reflux for twelve minutes. The deep red solution was acidified with glacial acetic acid, evaporated to dryness, made alkaline with dilute ammonia, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The brown resin was treated with ether and the insoluble brown impurities were removed by filtration. The filtrate, on evaporation, yielded a pale yellow amorphous residue (6 mg.), $\lambda_{max.}$ 320 mμ (ϵ 11,200), 285 mμ (ϵ 6,400); $\lambda^{0.01\ N\ NaOH}_{max.}$ 375 mμ (ϵ 8,600), 343 mμ (ϵ 6,600).

[2] We thank Mr. Murle Klohs of Riker Laboratories, Northridge, Calif., for comparison samples of germanitrine and germanidine.

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Veratrum Alkaloids XL*

The Structure of Escholerine, A Hypotensive Ester Alkaloid

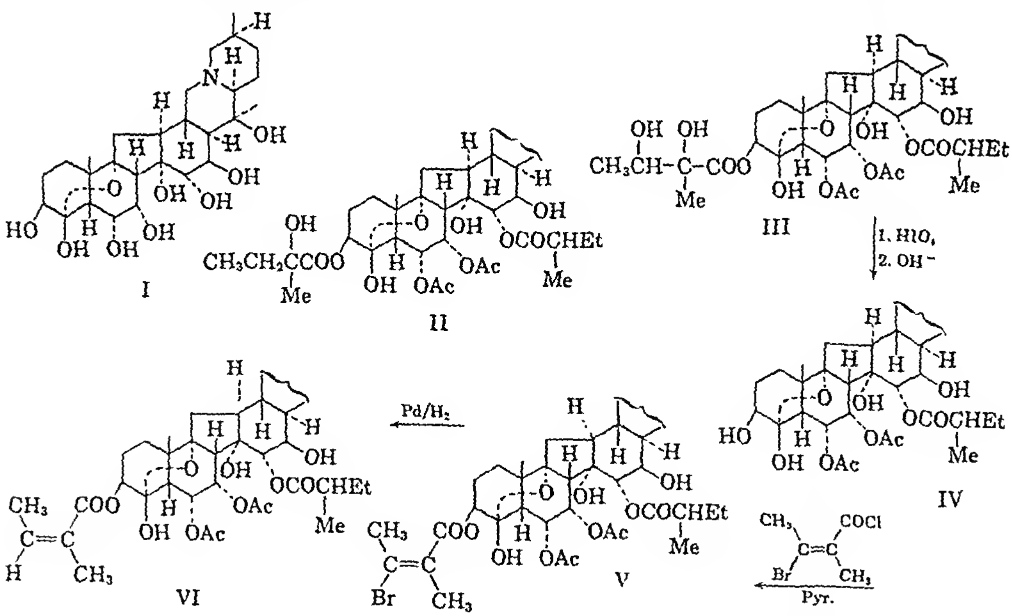
By S. MORRIS KUPCHAN and C. IAN AYRES

The structure of escholerine has been elucidated. Synthesis of protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate afforded a product identical with escholerine.

ESCHOLERINE is one of the main hypotensive principles of *Veratrum eschscholzii* Gray (1, 2). Hydrolysis of escholerine has afforded the known alkamine protoverine (I) (3, 4, 5), two mole equivalents of acetic acid, one mole equivalent of *l*-2-methylbutyric acid and one mole equivalent of angelic acid (1, 2). The positions of the four esterifying acids on the protoverine nucleus have now been established, thereby effecting the elucidation of the complete structure of escholerine.

A common structural feature of the two known naturally-occurring esters of protoverine, protoveratrine A (II), and protoveratrine B (III) is the attachment of acetates at positions C_6 and C_7 and of *l*-2-methylbutyrate at C_{15} (6, 7). Consequently, on a tentative biogenetic basis, one might expect a similar distribution of these three acids in escholerine. Furthermore, all the hypotensive protoverine esters studied to date possess a free (unacylated) hydroxyl group at C_{16}. Hence to test this hypothesis, the synthesis of protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (VI) was undertaken.

Protoverine 6,7-diacetate 15-*l*-2′-methylbutyrate (IV) can be readily prepared from protoveratrine B (7). Triester IV was treated with a limited amount of 3-bromoangeloyl chloride (8, 9) to yield protoverine 3-(3′-bromoangelate) 6,7-diacetate 15-*l*-2′-methylbutyrate (V) $[\alpha]_D^{23}$ $-16°$ (pyr.). The latter compound, on hydrogenation, afforded protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (VI), m. p. 235–236° (decompn.) $[\alpha]_D^{26}$ $-28°$ (pyr.). The angelic acid was shown to be attached to C_3 in the following manner. A chromic acid titration of the tetraester showed the presence of only one secondary hydroxyl group and C_4 was thereby excluded as a site for attachment of the angelic acid ester. Furthermore, the oxidation product, on treatment with alkali, afforded a diosphenol with spectral properties identical with those of the diosphenol from 16-dehydroprotoverine 3,4,6,7,15-pentaacetate (5). Therefore, the synthetic tetraester was protover-



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ine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (VI). The compound showed no depression in melting point on admixture with an authentic sample of escholerine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Ultraviolet absorption spectra were determined on a model 11 MS Cary recording spectrophotometer and 95% ethanol was used as solvent. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as a solvent.

Protoverine 3-(3′-bromoangelate) 6,7-diacetate 15-*l*-2′-methylbutyrate (V).—Protoverine 6,7-diacetate 15-*l*-2′-methylbutyrate [(7), 627 mg., m. p. 232–233° (decompn.)] was dissolved in pyridine (10 cc.), cooled to −10°, and treated with 3-bromoangeloyl chloride (0.2 cc.). The mixture was stirred until a clear yellow solution was obtained. The solution was allowed to come to room temperature and stand overnight. The pH was brought to 9 with dilute ammonia and the mixture extracted thoroughly with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to yield a brown resin (640 mg.). A paper chromatogram employing the procedure of Levine and Fischbach (10) [*n*-butyl acetate: *n*-butanol: formic acid (25:5:1 by volume)] indicated the formation of a new material in a yield of approximately 25%. The resin was chromatographed on Merck acid-washed alumina (15 Gm.). The column yielded to benzene, benzene-chloroform, and chloroform a series of yellow oils. When developed with chloroform-1% methanol, however, an amorphous material was obtained (110 mg.), $[\alpha]_D^{23}$ −16° (c 0.89, pyr.) whose paper chromatogram indicated that it was homogeneous in nature.

Hydrogenation of Protoverine 3,(3′-bromoangelate) 6,7-diacetate 15-*l*-2′-methylbutyrate: Synthesis of Escholerine (VI).—The amorphous 3,(3′-bromoangelate) (67 mg. of V. $[\alpha]_D^{23}$ −16°) was dissolved in 95% ethanol (6 cc.) containing sodium acetate (45 mg.) and added to a suspension of 10% palladium on charcoal (35 mg.) in 95% ethanol (4 cc.) that had previously been saturated with hydrogen. After two minutes the theoretical uptake of hydrogen was consumed and the hydrogenation was terminated. The catalyst was removed by filtration and the filtrate was acidified with glacial acetic acid and evaporated nearly to dryness. The residue was dissolved in water, basified with dilute ammonia, and extracted with chloroform. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to yield a resin which was crystallized from acetone-water to give colorless plates, m. p. 235–236° (decompn.); $[\alpha]_D^{26}$ − 28° (c 0.9, pyr.); yield, 30 mg. The melting point was not depressed on admixture with an authentic sample of escholerine.[1] The infrared spectra and paper chromatographic behavior of the respective samples were identical. A second crop of material (15 mg.) with m. p. 234–236° (decompn.) was also obtained.

Chromic Acid Oxidation of Escholerine.—Protoverine 3-angelate 6,7-diacetate 15-*l*-2′-methylbutyrate (escholerine, m. p. 234–236°, 15 mg.) in glacial acetic acid (5 cc.) was treated with 0.05 *N* chromic anhydride in 99.8% acetic acid (3 cc.). The solution was allowed to stand at room temperature. Aliquot parts titrated after one hour and three hours showed the consumption of 1.20 and 1.28 oxygen equivalents of chromic acid. An aliquot (4 cc.) taken after one hour was treated with excess sodium bisulfite to destroy the oxidizing agent, basified with dilute ammonia, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to give a resin (7 mg.). The resin was dissolved in 2 cc. of a solution of sodium hydroxide (100 mg.) in methanol (10 cc.) and heated under reflux for twelve minutes. The reaction mixture was acidified with glacial acetic acid and evaporated to dryness. The residue was dissolved in water, basified with dilute ammonia, and extracted with chloroform. Evaporation of the chloroform afforded an amorphous residue which showed $\lambda_{max.}$ 328 mμ (ϵ 12,700) and $\lambda_{max.}^{0.1 N\ NaOH}$ 381 mμ (ϵ 8,800).

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[1] We thank Mr. Murle Klohs of Riker Laboratories, Inc., Northridge, Calif., for a comparison sample of escholerine.

Veratrum Alkaloids XLI*

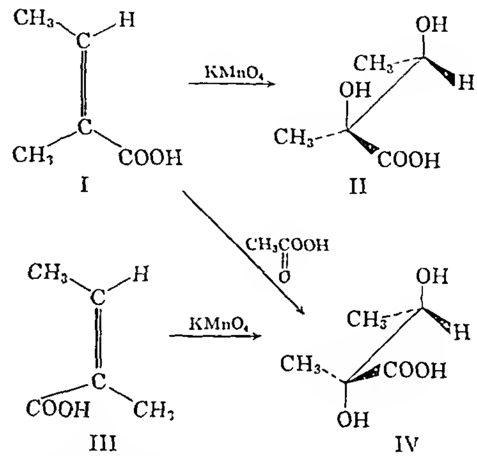
The Structure of Germbudine, A Hypotensive Ester Alkaloid

By S. MORRIS KUPCHAN and NORBERT GRUENFELD†

The structure of germbudine has been elucidated as germine 3-*d-threo*-2′,3′-dihydroxy-2′-methylbutyrate 15-*l*-2 -methylbutyrate (V). Oxidation of germbudine with periodic acid and subsequent alkaline hydrolysis afforded germine 15-*l*-2′-methylbutyrate (VIII).

GERMBUDINE is a minor hypotensive constituent of alkaloidal extracts of *Veratrum viride* (1). Alkaline hydrolysis of germbudine was reported to yield the known alkamine germine (IX), one mole equivalent of 2-methylbutyric acid, and one mole equivalent of high melting *d*-2,3-dihydroxy-2 methylbutyric acid.

Low melting *l*-2,3-dihydroxy-2-methylbutyric acid is one of the esterifying acids of neogermbudine and germitetrine, and high melting *d*-2,3-dihydroxy-2-methylbutyric acid occurs in germbudine and protoveratrine B (1–5). In 1953, Nash and Brooker (3) suggested that these two acids were diastereoisomers on the basis of their differing physical characteristics and the identification of acetaldehyde and pyruvic acid as common periodic acid cleavage products. In 1955, Myers, *et al.* (1), confirmed this hypothesis by synthesis of both acids. The racemic low melting diastereoisomer was synthesized by hydroxylation of tiglic acid (I) with dilute permanganate and was resolved to obtain the *l*-2,3-dihydroxy-2-methylbutyric acid identical with the acid isolated from natural sources (*levo* II). Hydroxylation of a double bond with permanganate is known to proceed in a *cis* fashion (6) and therefore the low melting diastereoisomer is the *erythro* isomer (II). The racemic high melting diastereoisomer was synthesized by hydroxylation of tiglic acid with peracetic acid and was not resolved. However derivatives of the racemic compound and dextrorotatory natural acid showed identical infrared spectra. The natural acid also had a melting point different from that of the synthetic *d-erythro acid*. Since peracetic acid is known to hydroxylate a double bond in a *trans* fashion (6) the high melting diastereoisomer is the *threo* isomer (IV). This assignment is supported by the observation that the latter racemic synthetic acid has the same melting point as the acid obtained on hydroxylation of angelic acid (III) with dilute permanganate (7).



Thus the esterifying acid of neogermbudine and germitetrine is *l-erythro*-2,3-dihydroxy-2-methylbutyric acid and the esterifying acid of germbudine and protoveratrine B is *d-threo*-2,3-dihydroxy-2-methylbutyric acid. These assignments render the chemical nomenclature of these hypotensive alkaloids more meaningful than the one based on melting points of the acids (1, 2, 8).

A common structural feature of most naturally-occurring diesters of germine is the attachment of the acyl groups at C_3 and C_{15}. Neogermbudine, which occurs alongside germbudine in *Veratrum viride*, has been shown to be germine 3-*l-erythro*-2′,3′-dihydroxy-2′-methylbutyrate 15-*l*-2′-methylbutyrate (VI) (2). Germbudine and neogermbudine possess very similar melting points, optical rotations, and infrared spectra (1). They also demonstrate very similar paper chromatographic behavior. The ethylene chloride-cellosolve acetate-pyridine system of Levine and Fischbach (9) proved adequate for their separation, but only after prolonged development.

On the basis of the similarity of physical constants, the possibility that germbudine could be a diastereoisomer of neogermbudine was formulated as a working hypothesis for the elucidation of its structure. Germbudine, in agreement with the postulation of a C_3, C_{15} diester and the consequent presence of only one free glycol group (that of the dihydroxy acid) consumed 0.85 mole

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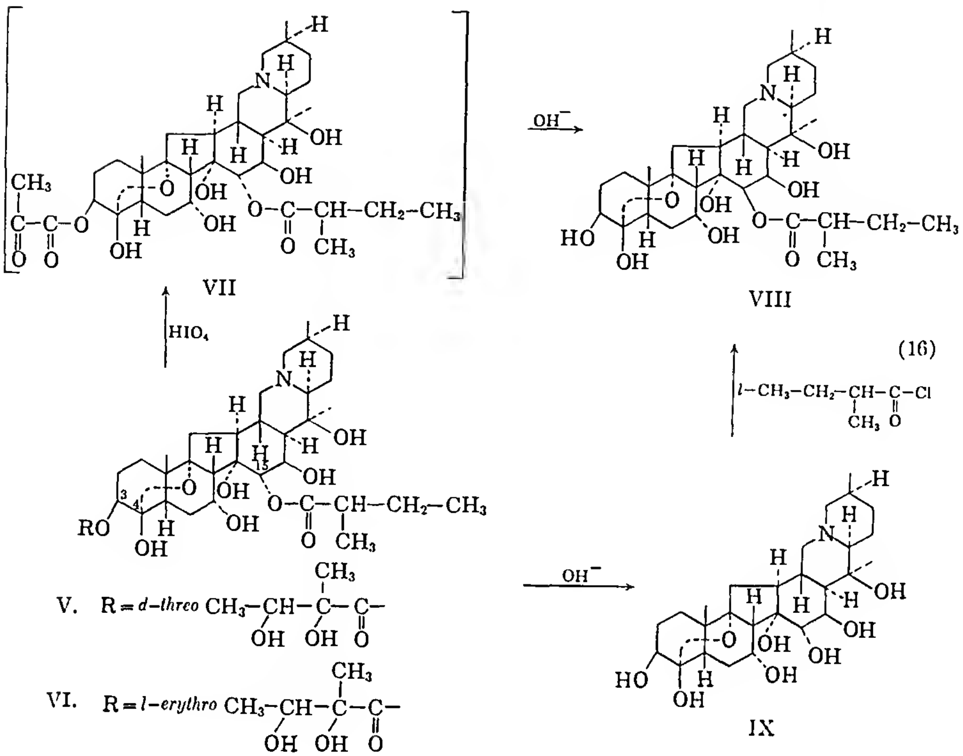

equivalent of periodic acid. The periodic acid titer did not distinguish between the C_3, C_{15} and the C_4, C_{15} diester structures. The presence of an acyl moiety at C_4 was deemed highly improbable on biogenetic grounds. No C_4 ester has been found among the more than twenty naturally-occurring derivatives of the veratrum alkamines veracevine, germine, protoverine, and zygadenine (2, 8, 10–14). Evidence in favor of the C_3, C_{15} structure was obtained by chromic acid titration of the diester. Germbudine demonstrated a chromic acid consumption which paralleled that of neogermbudine and indicated the presence of three secondary hydroxyl groups.

The specific location of each acyl group was determined by the selective removal of the *d-threo*-2,3-dihydroxy-2-methylbutyryl moiety by a procedure similar to that employed for the structure elucidation of protoveratrine B (13). Oxidation of germbudine with periodic acid at room temperature for three hours gave, as determined by paper chromatographic analysis, a quantitative yield of a product of higher R_f than the starting material [presumably germine mono-2-methylbutyrate mono-pyruvate (VII)]. Taking advantage of the facile base-catalyzed hydrolysis of pyruvate esters (13, 15), the periodic acid oxidation mixture was rendered alkaline with ammonia after decomposition of excess periodic acid with sodium bisulfite and was kept at room temperature for twenty minutes. Paper chromatographic analysis of the chloroform extracted product revealed partial conversion to a compound having the same R_f as germine-15-*l*-2′-methylbutyrate (VIII) (2, 16). Incomplete hydrolysis may have been due to the formation of a bisulfite addition compound at the ketone function of the pyruvate. Retreatment of the product with dilute ammonia led to complete hydrolysis. Crystallization from chloroform-ether gave a product shown by infrared spectrum, paper chromatographic, and melting point behavior to be germine 15-*l*-2′-methylbutyrate. Hence the 2-methylbutyric acid of germbudine is assigned the *levo* configuration, and germbudine is germine 3-*d-threo*-2′,3′-dihydroxy-2′-methylbutyrate 15-*l*-2′-methylbutyrate (V).

The degradation of neogermbudine by a sequence of steps paralleling that described for germbudine also gave germine 15-*l*-2′-methylbutyrate. This route was found to be more elegant than the sodium borohydride method employed earlier (2).

EXPERIMENTAL

Melting points are corrected for stem exposure. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as the solvent. Paper chromatograms were run by the descending technique employing Whatman No. 1 paper.

Paper Chromatography.—The procedure and solvent systems employed were similar to those described by Fischbach and Levine (9, 17) for an ascending technique. This method involves the use of prewetted pH 3.5 buffered paper and the detection of alkaloids by spraying with a chloroform solution of bromphenol blue. The solvent systems used were: (*A*) the solution prepared by adding 1 cc. of formic acid to the separated organic layer of the system *n*-butyl acetate-*n*-butanol-water (10:25:10); (*B*) cellosolve acetate-ethylene chloride-pyridine (10:15:1). System *B* was used for testing the homogeneity of germbudine,[1] neogermbudine,[1] and germine-15-*l*-2′-methylbutyrate. Separation of these alkaloids was successful on development for eighteen hours allowing solvent to run off the paper, as R_f values were relatively low. System *B*, development period of twelve hours, was utilized to follow periodic acid oxidations and degradations of germbudine and neogermbudine. System *A* was employed for differentiation of germine 15-*l*-2′-methylbutyrate and germine 3-*l*-2′-methylbutyrate (protoveratridine) (8).

Degradation of Germbudine to Germine 15-*l*-2′-Methylbutyrate.—Germbudine (V, 21 mg.), m. p. 159–161°, was dissolved in 5% acetic acid (5 cc.); 0.05 *M* periodic acid (15 cc.) and sufficient distilled water to dilute the solution to 25 cc. were added. The periodic acid consumption, as determined by titration of 1-cc. aliquots (18), was 0.85 mole equivalent after one and after three hours. The remainder of the solution was first treated with 7% sodium bisulfite (10 cc.) and then with sufficient 2 *N* ammonium hydroxide to bring the solution to pH 8.5–9. After standing at room temperature for twenty minutes, the ammoniacal solution was extracted with chloroform; the combined chloroform solution was washed with water, dried with sodium sulfate and evaporated to dryness under reduced pressure. The paper chromatogram of the reaction product revealed the absence of starting material; it also suggested the presence of germine 15-*l*-2′-methylbutyrate (VIII) and of a second product of R_f higher than that of germbudine [possibly germine 3-pyruvate 15-*l*-2′-methylbutyrate (VII)]. The reaction product was dissolved in 5% acetic acid (5 cc.) and a few drops of chloroform; sufficient 2 *N* ammonium hydroxide was added to bring the solution to pH 9. The mixture was kept at room temperature for twenty minutes and then extracted with chloroform. The chloroform extract was washed, dried, and evaporated to dryness to give a product (15 mg.) which proved homogeneous on paper chromatographic analysis and showed R_f identical to that of germine 15-*l*-2′-methylbutyrate (VIII). The crude product was dissolved in a drop of chloroform, excess ether was added, and the flocculent precipitate which formed immediately was removed by filtration. White elongated prisms (9.5 mg.) separated from the filtrate on standing overnight and were recrystallized twice from chloroform-ether, yield 5 mg., m. p. 222–224° (decompn.). No melting point depression was observed on admixture with an authentic sample of germine 15-*l*-2′-methylbutyrate and the infrared spectra of the respective samples were identical (2, 16). The paper chromatographic behavior of the product was the same as that of the authentic sample and different from that of protoveratridine, germine 3-*l*-2′-methylbutyrate (8).

Degradation of Neogermbudine to Germine 15-*l*-2′-Methylbutyrate.—Neogermbudine (VI, 19.4 mg.), m. p. 149–152° (decompn.), was submitted to the degradation procedure described for germbudine. It showed a periodic acid uptake of 1.0 mole equivalent after one hour and 1.1 mole equivalents after three hours. Workup of the periodic acid oxidation mixture led to the isolation of germine 15-*l*-2′-methylbutyrate (VIII); first crop, 2.5 mg., m. p. 220–222° (decompn.); second crop, 5 mg., m. p. 215–218° (decompn.).

Chromic Acid Titrations.—The alkaloid (*ca.* 1×10^{-2} m*M*) was dissolved in glacial acetic acid (3 cc.) and treated with a solution of chromic anhydride in acetic acid containing 0.2% of water (2 cc., 0.055 *N*). The oxidation was allowed to proceed at room temperature and 2-cc. aliquots were withdrawn after reaction periods of one and two hours. The chromic acid in oxidation mixtures and blank was reduced with 10% potassium iodide solution (5 cc.) and the released iodine was titrated with standard 0.01 *N* sodium thiosulfate. The results of titrations run on identical molar concentrations of germbudine, neogermbudine, and germine 15-*l*-2′-methylbutyrate are given in Table I.

TABLE I.—CHROMIC ACID TITRATIONS

Alkaloid	Oxygen Equivalents of Chromic Acid Consumed: Theoretical	Found, 1 hr.	Found, 2 hr.
Germine 15-*l*-2′-methylbutyrate (VIII)	3	3.0	3.3
Neogermbudine (VI)	3	2.9	3.3
Germbudine (V)	3	3.0	3.3

[1] We thank Dr. G. S. Myers of Ayerst, McKenna and Harrison Ltd. for authentic samples of germbudine and neogermbudine.

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An Improved Colorimetric Method for the Determination of Meprobamate in Biological Fluids*

By ALLAN J. HOFFMAN† and B. J. LUDWIG

A colorimetric procedure is described for the microdetermination of meprobamate in biological fluids. Meprobamate is extracted from plasma or serum using mixed chloroform-carbon tetrachloride solvent, and color development is effected by treatment with *p*-dimethylaminobenzaldehyde and antimony trichloride in acetic anhydride. The color intensity measured at 550 mμ is proportional to the concentration over the range of 0.5 to 10 mcg. A suitable modification of the extraction procedure for use with urine specimens is also described, which permits separation of meprobamate from the higher concentration of endogenous interfering substances present in urine. Determination of meprobamate concentrations in blood specimens from normal adult subjects indicates that a peak concentration of this drug is attained about two hours after oral administration.

In earlier publications (1–7) it has been shown that orally administered meprobamate is readily absorbed into the blood stream from the gastrointestinal tract of experimental animals and of humans and is excreted in the urine in an unchanged form, as a hydroxylated derivative, and in the form of a yet unidentified glucuronide. The analytical methods used in connection with these studies generally employed nonspecific colorimetric procedures or depended solely or partly on chromatographic separation. In some animal studies C^{14}-labeled meprobamate was utilized as a basis for analysis. None of the published methods permits a rapid and practical estimate of meprobamate content in blood and urine in the concentrations normally encountered and in the presence of endogenous interfering substances.

Ludwig and Hoffman (8) described a chemical reaction between meprobamate and certain aldehydes which served as a basis for a colorimetric procedure for the determination of this drug. It is the purpose of this publication to present a simplified and improved modification of this highly specific method and to show its application to a series of blood concentration and urinary excretion studies conducted on adult human subjects.

Meprobamate develops an intense red-violet color when reacted with certain aldehydes and anhydrous metallic salts in the presence of acid dehydrating agents.[1] An examination of the action of a number of such combinations led to the selection of *p*-dimethylaminobenzaldehyde and antimony trichloride in acetic anhydride as the reagents of choice from the viewpoint of prompt and consistent color development, permanence of color intensity, and adherence to Beer's law. This color reaction is specific for unsubstituted amides. By proper selection of extraction solvent interference from naturally-occurring amides, urea, etc., can be reduced to an insignificant minimum. Because of the greater water solubility of both hydroxymeprobamate and the glucuronide conjugate of this drug, the extraction procedure as described does not remove these metabolic products of meprobamate from the usual body fluids. When carried out according to the procedure given below, this method will permit the accurate determination of meprobamate in urine, plasma, serum, or spinal fluid in concentrations from 0.5 to 10 mcg. per ml.

EXPERIMENTAL

Reagents and Apparatus.—Reagent grade materials are used throughout and all solvents are redistilled through a packed column before use. Low actinic containers fitted with glass stoppers or Teflon-lined closures are recommended.

Antimony Trichloride-Acetic Anhydride Reagent (ATA Reagent).—A stock solution consisting of a saturated solution of antimony trichloride in chloroform (approximately 25% w/v) is first prepared by warming the mixture on a hot plate to solution, cooling, and filtering through Whatman 41H paper. ATA reagent is prepared by combining four volumes of this stock solution with one volume of acetic anhydride. This reagent is stable for two days when stored under refrigeration.

***p*-Dimethylaminobenzaldehyde Solution (DMB Reagent).**—A 1% w/v solution of the aldehyde in benzene. When stored in the refrigerator, this solution is stable for about one month.

Acetic Acid-Acetone Reagent (AAA Reagent).—A mixture of three volumes of reagent grade acetone and one volume of glacial acetic acid.

Mixed Solvent.—A mixture of equal volumes of carbon tetrachloride and chloroform.

Procedure.—One milliliter of plasma or serum is transferred to a 40-ml. glass-stoppered centrifuge tube. Two drops of 28% ammonium hydroxide, 2

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The authors wish to acknowledge the valuable technical assistance of Mr. John Gatto of these laboratories.

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[1] The nature of the colored compound has not been investigated. Since the intense color development characteristic of this reaction apparently requires the participation of certain cations in the form of their anhydrous halides ($SbCl_3$, $SiCl_4$, $ZnCl_2$, etc.) it is unlikely that the colored compound possesses the simple anil structure postulated by Foley, Sanford, and McKennis (9) for a similarly colored compound formed from aniline and furfural.

drops of saturated potassium chloride solution, and 25 ml. of mixed solvent are added. A reagent blank consisting of 1 ml. of distilled water, ammonium hydroxide, potassium chloride solution, and mixed solvent is run simultaneously. The tubes are shaken vigorously for five minutes, then centrifuged for ten minutes. The organic layer is then carefully removed with a 30-ml. hypodermic syringe fitted with a 10-cm. needle and passed into a second centrifuge tube through a small pledget of Pyrex glass wool (previously extracted with chloroform). The glass wool is rinsed with 5 ml. of mixed solvent and the combined extract and rinse solutions evaporated to dryness by immersing the tube in an oil bath at 80°. The tubes are rinsed down with a few ml. of acetone and the heating continued until the tubes are thoroughly dried. Color development is then effected by adding 0.2 ml. of AAA reagent followed by 0.2 ml. of DMB reagent and mixing. One ml. of ATA reagent is pipetted into the tubes, the contents mixed, the tubes stoppered and placed immediately in a 50° water bath, and held at this temperature for exactly ten minutes. The tubes are removed and cooled by immersing in cold water and then diluted with 1 ml. of benzene. The colored solutions are transferred to Corex glass-stoppered absorption cells and the absorbance measured using a Beckman DU spectrophotometer at a wavelength of 550 mμ. A graph of the absorbance as a function of wavelength is shown in Fig 1. Solutions of meprobamate in distilled water carried through the complete procedure as described are used to establish the calibration curve. The absorbance is directly proportional to the concentration over the range of 0.5–10 mcg. of meprobamate. Color intensity remains constant for at least one hour.

Conformity with Beer's Law.—A solution of meprobamate in water was prepared and aliquots containing 0, 2, 5, and 10 mcg. were diluted to 1 ml. with distilled water in one series and with normal human plasma in another series and treated in accordance with the above procedure. The absorbances of the colored solutions were read at 550 mμ against the prepared blank and were plotted against their respective concentrations (Fig. 2). The absorbances of solutions containing 0 to 10 mcg. of meprobamate gave a straight line relationship indicating conformance with Beer's law. The agreement between the distilled water and the plasma values at each concentration established that interfering components in plasma had been satisfactorily eliminated using this extraction procedure.

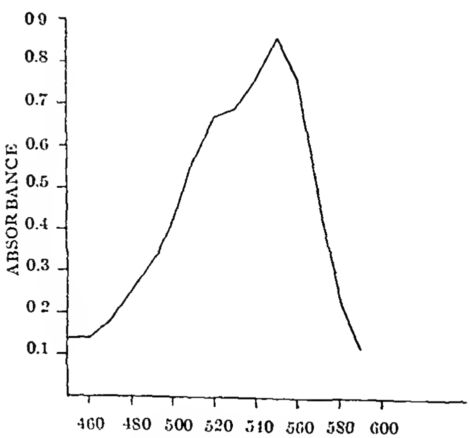

Fig. 1.—Absorption spectrum of meprobamate, *p*-dimethylaminobenzaldehyde and antimony trichloride color reaction. Determined with Beckman DU spectrophotometer using 1-cm. Corex cells, slit width 0.025 mm.

Similar studies made on human serum resulted in comparable recoveries of meprobamate and the procedure without modification was found to be suitable for spinal fluid studies. The presence of hemoglobin in plasma or serum had no adverse effect on this analytical method.

Accuracy and Precision.—To establish the accuracy and precision of this procedure, eight determinations were made at each of three concentrations in both water and normal human plasma. The precision at each level of concentration for each series is given in Table I.

TABLE I.—RECOVERY OF MEPROBAMATE FROM WATER AND HUMAN PLASMA

Meprobamate Added mcg./ml.	Absorbance, 550 mμ (Mean of 8 Determinations) Water	Plasma	Standard Deviation $SD = [1/N(x-\bar{x})^2]^{1/2}$ Water	Plasma
2.0	0.158	0 156	0.0127	0.0155
5.0	0 371	0.379	0.0158	0.0166
10.0	0.734	0.741	0 0280	0.0305

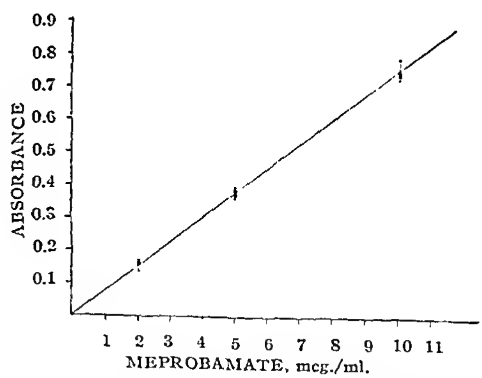

Fig. 2.—Absorbance of meprobamate, *p*-dimethylaminobenzaldehyde and antimony trichloride color reaction as a function of concentration: O water; ● plasma. Vertical lines denote magnitude of standard deviation for plasma determinations.

Analysis of Urine Specimens.—Urines of subjects receiving meprobamate collected during the twenty-four-hour period following administration generally contain meprobamate in amounts several-fold times the blood concentration of this drug, necessitating a 1:5 or 1:10 dilution prior to extraction. These dilutions contain interfering amounts of urea and other substances, and must be subjected to an additional treatment step before color development is attempted.

The residue obtained on evaporation of the

clarified mixed solvent extract is heated for fifteen minutes at 50° with 1 ml. of 0.2 N hydrochloric acid. It is then made alkaline with ammonia, potassium chloride solution added, and the procedure carried through as described above.

Application to Blood and Urine Specimens—Six healthy human adults were administered two 400-mg. tablets of meprobamate and blood specimens taken at one, two, four, six, eight, and twelve hours were analyzed for their meprobamate content. The individual values and a curve representing an average are plotted in Fig. 3. The peak blood concentrations occurred at about two hours, followed by a steady decline for ten hours or longer. At peak concentration, it is estimated that the total content of meprobamate in blood of these adult human subjects was about 15% of the ingested dose.

The meprobamate content of urines from several normal adult humans ingesting similar quantities of this drug were determined using the procedure as modified for urine analysis. In general, approximately 10% of the drug was excreted unchanged in the urine during the twenty-four-hour period following ingestion and a few additional per cent was excreted in the subsequent twenty-four-hour period. These findings are in agreement with those noted by others (1, 4, 5).

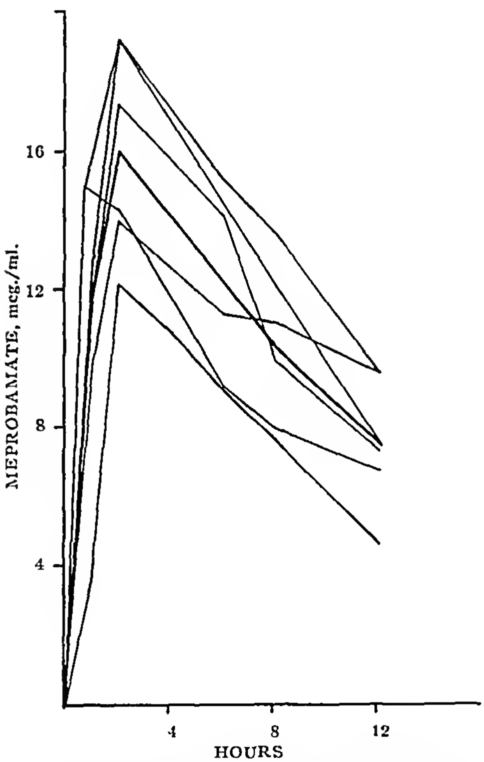

Fig. 3.—Individual plasma concentrations of meprobamate for adult human subjects receiving two 400-mg. tablets. Heavy line represents average of six individual studies.

DISCUSSION

A comparison of these human blood concentration values with those of experimental animals is of interest. Laboratory mice receiving substantially larger oral doses of meprobamate (300 mg./Kg.) showed a peak blood content of 245 mcg./ml. about one-half hour after oral administration. This result is consistent with the findings of Walkenstein, *et al.* (4), who reported that the peak C^{14} content of lung, liver, heart, spleen, and kidney occurred thirty minutes after oral administration of C^{14}-labeled meprobamate (400 mg./Kg.). In each case the rate of disappearance of meprobamate approximated that observed in humans. Whether this difference in time required to attain peak blood concentration is a function of the magnitude of the dose administered, or represents a significant species difference has not been elucidated.

A number of blood specimens from subjects who had consumed quantities of meprobamate far in excess of that usually prescribed were found to contain 100 to 200 mcg./ml. One such patient who allegedly consumed 12 Gm. of this drug had serum and spinal fluid levels of 182 and 50 mcg./ml., respectively. The urine of this patient contained 0.25% meprobamate.

SUMMARY

Meprobamate reacts with p-dimethylaminobenzaldehyde and antimony trichloride in the presence of acetic anhydride to give a red-violet color having an absorption maximum at 550 mμ. Concentrations of meprobamate in biological fluids have been accurately determined by use of a suitable extraction procedure and application of this color reaction.

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A Pharmacognostical and Phytochemical Study of *Maianthemum Canadense* (Desf.)*

By C. W. DICKERSON†

A number of valuable plant constituents have been obtained from the Lily family and, therefore, *Maianthemum Canadense* (Desf.) was deemed worthy of investigation. The taxonomy, distribution, and habitat of *Maianthemum Canadense* (Desf.) are recorded herein. A study of the morphological and histological features of the leaf, root, and whole plant powder, and the phytochemical and pharmacological properties of whole plant extracts are also described.

Examination of the scientific literature pertaining to *Maianthemum Canadense* (Desf.) revealed that the pharmacognostical, phytochemical, and pharmacological properties of this plant have not been recorded.

The plant was originally described by Réne Desfontaines, who gave it the name *Maianthemum*, derived from the Latin, Maius meaning May; and the Greek, anthemon meaning flower (1). The available information does not show any chemical analysis of *Maianthemum Canadense* (Desf.), but in 1907 Eldridge and Liddle (2) published their phytochemical study of *Smilacina racemosa* (Desf.), which closely resembles *Maianthemum*. Their results were as follows: the berries contain tartaric and oxalic acids, possibly in the form of potassium salts; tannin; levulose; and also some dextrose. The seeds contain levulose; fixed oil in the form of olein and palmitin; and some gum.

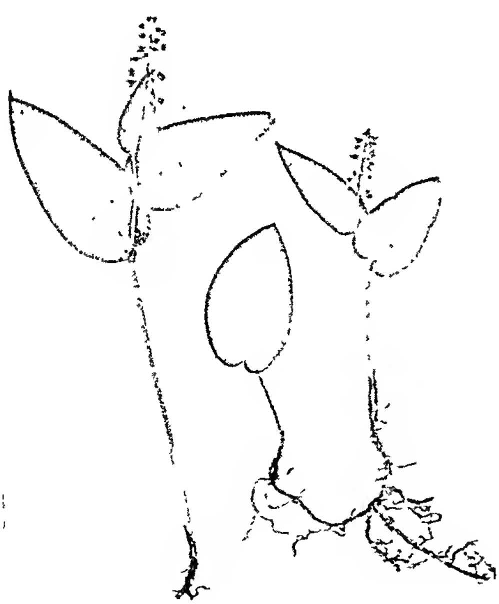

Fig. 1.—Flowering plants of *Maianthemum Canadense* (Desf.).

TAXONOMY

Maianthemum Canadense (Desf.) is a member of the family *Liliaceae* (Lily family). The most widely used synonym is wild lily-of-the-valley along with other common names of Canada mayflower and bead ruby, which are recorded by E. H. Hausman (3). According to A. B. Lyons (4) it is classified as follows: *Unifolium Canadense* (Desf.) Green (*Maianthemum Canadense* (Desf.) and *Smilacina bifolia*, var. *Canadensis* A. Gray). N. L. Britton, and A. Brown (5), and M. L. Fernald (1) also give false or wild lily-of-the-valley and two-leaved Solomon's seal as the common names. The Index Kewensis (6) records several names that have been given in the past: *Bifolium* by Gaertn., Mey, and Scherb.; *Maia* by Salisb.; *Sciophila* by Wibel; *Styandre* by Raffin ; and *Unifolium* by Hall., Enum., Stirp., and Helv.

MORPHOLOGY

The description of the plant found in Mecosta County, Big Rapids, Michigan, follows the description given in Gray's Manual of Botany (1), "extensively creeping, the freely-forking filiform rhizomes bearing stalked tuberous enlargements; a single cordate leaf accompanying the flowering stem; the latter erect, glabrous, 0.5–2.5 dm. high, bearing two or three glabrous leaves; lower cauline leaf 2–10 cm. long; flowers sweetly fragrant, the perianth segments about 2 mm. long." See Fig. 1.

DISTRIBUTION AND HABITAT

Maianthemum Canadense (Desf.) is found abundantly in wooded areas and recent clearings, in sandy humus soil. It often ascends to the subalpine areas of Labrador, south to Newfoundland, New England,

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Long Island, Delaware, Pennsylvania, upland to Georgia, Tennessee, and Iowa

In Michigan it is widely distributed and found in moist wooded areas throughout the upper and lower peninsula

EXPERIMENTAL

Leaf.—Two methods of microscopical study were used The use of a microtome was employed and the usual preparation of the specimen was followed The mountings obtained by the microtome method were not satisfactory for drawing purposes, therefore fresh cuttings were made and mounted in chloral hydrate water These cuttings were observed at 10× and 43×

The leaf of *Maianthemum Canadense* (Desf) is typical of the Lily family The upper and lower epidermis has a striated cuticle with one row of short palisade cells, and is isodiametrical The spongy parenchyma is compact with a few of the cells containing calcium oxalate raphides Surrounding the vascular bundle is a ring of fibers (in older leaves) and one row of collenchyma cells, particularly at the lower surface of the leaf Representing the conducting tissues are small tracheae and tiny sieve tubes The cells of the upper and lower epidermis are elongated with wavy walls, having stomatal apparatuses only on the lower epidermis surrounded by four, or rarely by five, neighboring cells See Figs 2 and 3

Rhizome.—Epidermis consists of elongated cells accompanied by one row of collenchyma Filling the parenchyma of the pericycle are small starch

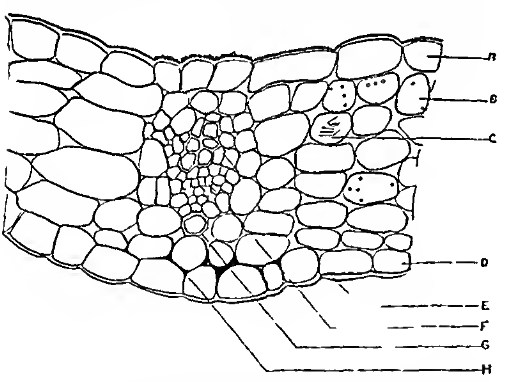

Fig 2—Cross section of leaf *A*, Upper epidermis, *B*, palisade cells, *C*, spongy parenchyma with calcium oxalate raphides, *D*, lower epidermis, *E*, tracheae, *F*, sieve tissue, *G*, bast fiber, *H*, collenchyma

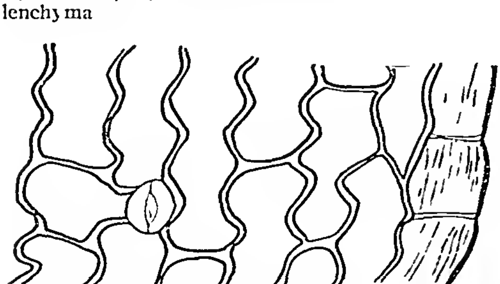

Fig 3—Lower epidermis with stoma and striated marginal epidermal cells

grains, the smallest being 15 μ, the largest 37 5 μ with an average of 23 2 μ, and calcium oxalate raphides in bundles from 22 5 to 45 μ with an average of 33 8 μ The endodermis is slightly lignified, consisting of one or, in some cases, two rows of cells The tracheae are arranged in groups surrounding the pith See Fig 4

Powdered Root and Rhizome—The powder is characterized by numerous small starch grains calcium oxalate raphides, fragments of tracheae, sclerenchyma fibers, and parenchyma

Powdered Leaf.—The identifying features of the powdered leaf are the calcium oxalate raphides, the characteristic epidermal cells, and the striated cuticle of those epidermal cells at the margin of the leaf See Fig 5

Phytochemistry—Collections of the whole plant were made in and around the Big Rapids, Michigan area during the latter part of April and May 1957 The material collected was dried at room temperature for three days before grinding in a hammer mill to a fine powder This powder proved to be very irritating to the nasal mucosa

Preliminary chemical tests were conducted on water infusions of the material collected and the following results tabulated

Substance	Reagent	Result
Alkaloids	$AuCl_3$ T S	−
	Mayer's reagent	−
	Picric acid T S	−
Tannins	$FeCl_3$ T S	+
Reducing substances (sugars, glycosides, and saponins)	Fehling's T S	+
Saponins	2% Rat blood suspension	+

Since the preliminary tests indicated the presence of saponins, further investigation dealt with the isolation of this type of compound

Twenty grams of the powdered plant were placed in Soxhlet extraction thimbles and extracted with petroleum ether for forty-eight hours, yielding 0 40 Gm of a green, waxy, resinous material (All yields indicated have been evaporated and desiccated to constant weight) The solvent was then changed to ethyl ether and extraction continued for forty-eight additional hours This solvent yielded 0 43 Gm of a green semioily material The thimbles were then placed in 300 ml of sterile distilled water containing a small amount of chloroform to retard mold growth After forty-eight hours of soaking, the thimbles were removed and the solution vacuum filtered through pumice The filtrate was then passed through 1 × 10 inch columns of Alcoa activated alumina which adsorbed a large portion of the coloring matter and other unwanted impurities The filtrate obtained was then evaporated without the use of heat in a vacuum desiccator until crystallization took place The crystals were then filtered out, yielding 0 06 Gm (0 3%) of a yellow crystalline material plus a yellow amorphous substance Varying amounts of ethyl alcohol and isopropyl alcohol were passed through the alumina columns following the aqueous extract, in order to determine the extent to which the extract was adsorbed on the alumina

Attempts to isolate the active principle by extraction with 95% ethanol and subsequent purifica

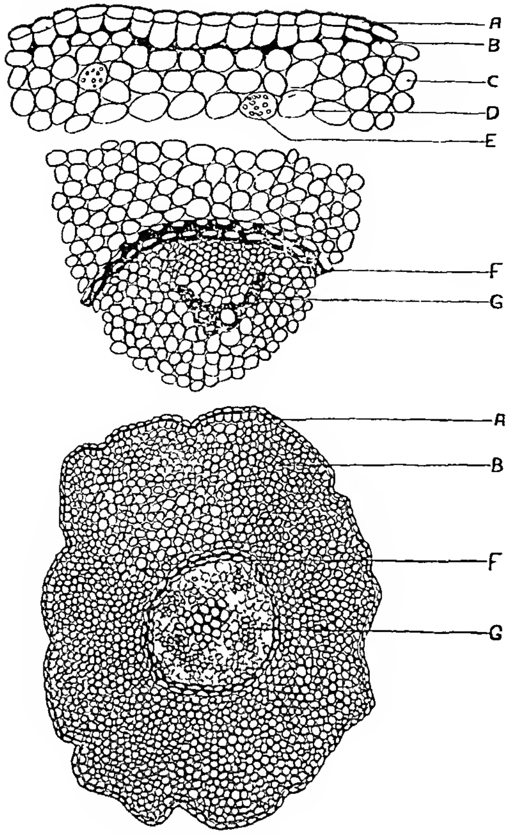

Fig 4 Top—Cross section of rhizome in high power *A*, Epidermis; *B*, collenchyma, *C*, peri cycle; *D*, parenchyma with calcium oxalate raph ides, *E*, parenchyma with starch grains; *F*, endoderm, *G*, tracheae

Bottom—Cross section of rhizome in low power, *A*, Epidermis, *B*, collenchyma, *F*, endoderm, *G*, tracheae

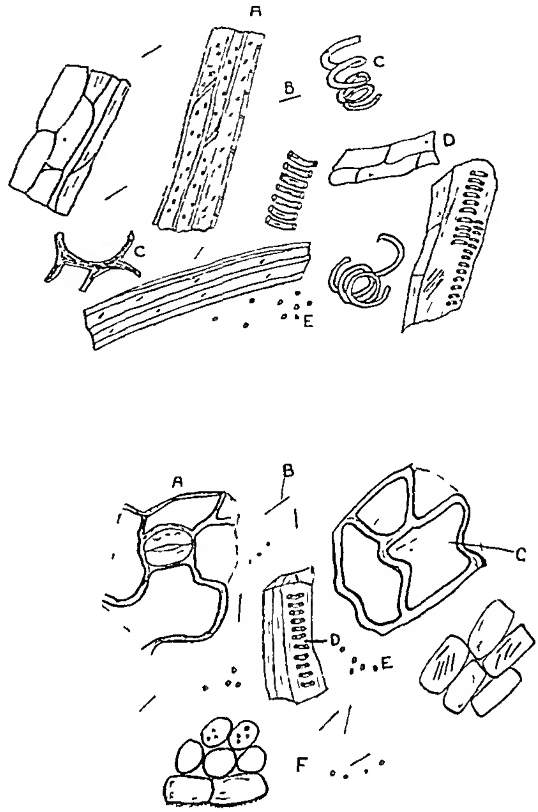

Fig. 5 Top—Powdered root and rhizome *A*, Fibers; *B*, calcium oxalate raphides, *C*, tracheae, *D*, parenchyma; *E*, starch grains.

Bottom—Powdered leaf. *A*, Lower epidermis with stoma; *B*, calcium oxalate raphides, *C*, upper epidermis; *D*, fragment of vascular bundle, *E*, chlorophyll grains, *F*, lower epidermis with spongy parenchyma

tion of this extract with lead subacetate were unsuccessful

Column chromatography was also attempted in the separation of the active constituents from the watery extract, but they were not adsorbed on the column and percolated through it, leaving this method advantageous only in purification

The two portions isolated were then tested for their hemolytic effect on rat blood The crystals proved to be some unpure, unidentified substance with no hemolytic properties, but the amorphous substance caused extensive hemolysis

PRELIMINARY STUDY OF PHARMACOLOGIC ACTIVITY

The water extract was tested for its effect on spontaneous contractions of the isolated ileum of rats and guinea pigs Doses of 0 5 ml (50 ml bath) produced an increase in tone, but had no effect on either rate or amplitude of contractions Larger doses of 1 0 ml and 2 0 ml resulted in a greater increase in tone

The same extract was tested for its effect on blood pressure The blood pressure was recorded by direct cannulation of the carotid artery and injections were made into the femoral vein Average responses were as follows·

Animal	Dose	Effect
Cat	0 2 ml /Kg	No effect
Cat	1 0 ml /Kg	Transient fall to 80% of normal
Rat	1 0 ml /Kg	Transient fall to 75% of normal
Rat	2 0 ml /Kg	Transient fall to 50% of normal

From these results it was impossible to ascertain definite pharmacological properties Additional pharmacologic investigations are in progress

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Quantitative Determination of Flavanones in Citrus Bioflavonoids by Potassium Borohydride Reduction*

By KENNETH M. ROWELL and DONALD H. WINTER

Flavanones reduced with potassium borohydride and acidified with hydrochloric acid develop red to violet colors. This reaction forms the basis for a quantitative spectrophotometric method for the determination of flavanones. The procedure outlined is sufficiently sensitive to determine quantitatively solubilized flavanones at concentrations of 0.02 mg./ml. Determinations of flavanone content of typical pharmaceutical formulations containing citrus bioflavonoids demonstrate complete recovery of added bioflavonoids. The method is rapid and the results are reproducible.

Definitive papers dealing with molecular structure, chemical properties, and biogenesis of flavanones have been written by T. A. Geissman, *et al.* (1, 2). Physiological properties, capillary and stress effects primarily, have been reported by Scarborough and Bacharach (3), Levitan (4), Martin (5), to mention a few.

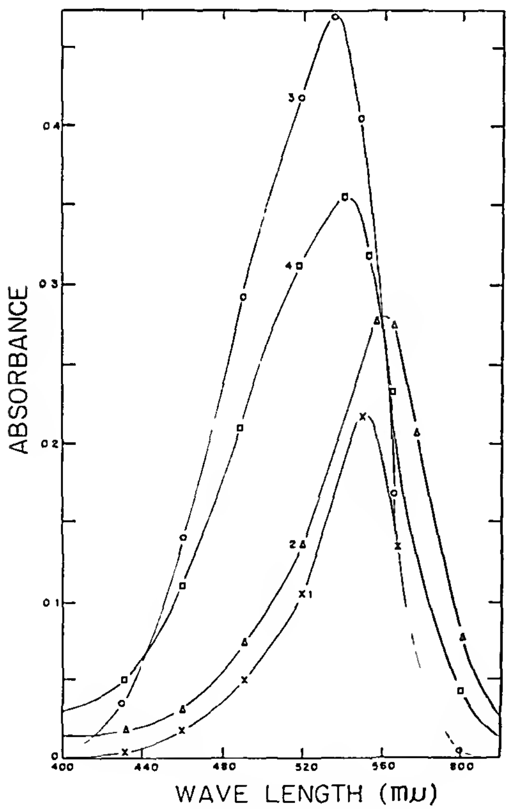

Fig. 1.—Absorption spectra of colored substances formed by reduction and subsequent acidification of the flavanones. (1) Naringin; concn. 2.08 γ/ml., max. 552 mμ; (2) Hesperidin: concn. 4.33 γ/ml., max. 560 mμ; (3) Naringenin: concn. 2.08 γ/ml., max. 535 mμ; and (4) Hesperetin: concn. 2.08 γ/ml., max. 540 mμ. Solvent: Acid reagent, 10-mm. Pyrex cells used.

* Received October 21, 1958, from the Orange Products Division, Sunkist Growers, Ontario, Calif

Methods for quantitative estimation of flavanones, chief group of bioflavonoids in citrus, have been generally unsatisfactory. The gravimetric as well as previous colorimetric methods lack specificity and convenience especially when applied to finished pharmaceuticals containing small amounts of flavanones compared to other ingredients. Colorimetric methods based on the cyanidin reaction (1) appeared to warrant careful consideration. Earlier attempts to adapt this reaction to the quantitative determination of flavanones have been of limited utility because of the difficulty in controlling the large amount of heat generated during magnesium reduction, with consequent variation in the colors produced. It was, therefore, desirable to find a better method of reduction of the flavanones with controlled color development.

In recent years the complex saline hydrides have become commercially available and have been shown to possess specific reducing properties (6, 7). Bauer, Birch, and Hillis (8) reported the reduction of flavanone with sodium borohydride yielded *β* 4-chromanol, m. p. 146°. These investigators also reported the flavonols rutin and quercetin were not reduced by sodium borohydride. Horowitz (9) tested many flavonoids and found only the flavanones produced red to violet colors when reduced with $NaBH_4$ and acidified with hydrochloric acid. He used $NaBH_4$ followed by hydrochloric acid as a spray reagent to locate flavanone spots separated by paper chromatography.

EXPERIMENTAL

Selection of Reducing Agent.—Preliminary experimentation with sodium borohydride and potassium borohydride for the reduction of flavanones in alcoholic and aqueous solutions showed that little heat was liberated during the reduction. It was also found that reduction to form a stable colorless compound could be accomplished by a noncolor-producing acid, e. g., acetic acid, enabling a final well-controlled color development with hydrochloric acid. Because potassium borohydride is non-

hygroscopic and reacts less vigorously in an aqueous system than sodium borohydride, it proved the more satisfactory metallic hydride. In addition, uniform results were obtained using various lots of potassium borohydride.

Apparatus.—Absorbance measurements were made with a Beckmann model B spectrophotometer using 10-mm. Pyrex cells. Glassware used consisted of Normax volumetric pipets and flasks, Pyrex test tubes (20 × 150 mm.) with polyethylene stoppers. A water bath with a temperature 20° ± 0.5° was used for the adjustment of volumes and temperature control.

Reagents.—Potassium borohydride, 97+%, (Metal Hydrides Co., Beverly, Mass.). "Acid reagent": prepared by diluting 5 ml. of hydrochloric acid, (reagent, A. C. S.) to 100 ml. volume with glacial acetic acid, (reagent, A. C. S.). Sodium hydroxide solution, 0.1 *N*. Acetic acid solution, 0.5 *N*.

Preparation of Standard Flavanone Samples.—Flavanone glycosides and their corresponding aglycones were purified from commercially available materials by appropriate procedures.

Hesperidin (*Hesperetin-7-rhamnoglucoside*).—Colorless needles melting at 261–263° with decompn. (10, 11), gravimetric purity 98+%.

Hesperetin (*3', 5, 7,-Trihydroxy-4'-methoxy Flavanone*).—Colorless plates melting at 232–234° with decompn. (12).

Naringin Dihydrate (*Naringenin-7-rhamnoglucoside*).—Colorless needles melting at 170–172° with decompn. (13), gravimetric purity 98+%.

Naringenin (*4', 5, 7-Trihydroxy Flavanone*).—Colorless needles melting at 249–251° with decompn. (13).

ANALYTICAL PROCEDURE

Preparation of Flavanone Solutions.—Weigh 50 mg. of the flavanone (100 mg. in the case of hesperidin) into a 100-ml. volumetric flask. Solubilize the flavanone by adding 10 ml. of 0.1 *N* sodium hydroxide. Adjust the volume to 100 ml. with distilled water. Prepare final solution by diluting 1:10 for hesperidin; 1:20 for hesperetin and naringin; 1:40 for naringenin, with distilled water.

Reduction. Pipet 5 ml. of the final diluted flavanone solution into a test tube (20 × 150 mm.) containing 50 mg. of potassium borohydride. When the potassium borohydride has dissolved, add dropwise 5 ml. of 0.5 *N* acetic acid to reduce the flavanone.

Color Development.—Into a polyethylene-stoppered test tube pipet 5 ml. of "acid reagent." Pipet 1 ml. of the reduced flavanone material into the "acid reagent" tube. Shake and place in the 20° water bath. The color which develops is read against a blank prepared just prior to reading and consists of 1 ml. of the reduced material plus 5 ml. of glacial acetic acid. It is necessary to prepare the blank just prior to reading because the reduced aglycones produce a small amount of color upon long standing in glacial acetic acid. The holding time for color development varies with the flavanone being determined. When assaying hesperidin, the maximum color is read at eighteen minutes; for naringin and naringenin, thirty minutes; and hesperetin five minutes. Transfer the colored solution just before the maximum intensity has been reached and record maximum absorbance at the predetermined wavelength for the flavanone being assayed. See Figs. 1 and 2.

Adherence to Beer's Law.—Beer's law is in effect from the concentrations 0 to 25 mcg. per ml. for hesperetin, naringin, and naringenin. A good linear relationship is shown for hesperidin from 0 to 100 mg. per ml. concentration, see Fig. 3.

Sensitivity.—Quantities of flavanones as low as 5 mcg. per ml. of solution (30 mcg. per ml. of hesperidin solution) can be determined by the potassium borohydride reduction method, see Fig. 3. The detectability level of the magnesium-hydrochloric acid reduction method is about 100 mcg. per ml. of ethanol (1). Thus, it appears that this test could be used to measure the amount of chromatographically isolated flavanones.

Reproducibility.—The reproducibility of results on several flavanones is shown in Table I. The absorbance values were obtained on individually reduced samples and illustrate the reproducibility of both the reduction and the color development.

Statistically, the standard deviations for the

TABLE I.—ABSORBANCE VALUES ON REPLICATE ANALYSES

Test No.	Naringenin	Hesperetin	Hesperidin	Naringin
1	0.260	0.375	0.300	0.224
2	0.258	0.372	0.300	0.225
3	0.260	0.375	0.299	0.223
4	0.260	0.372	0.299	0.222
5	0.261	0.378	0.298	0.222
6	0.259	...	0.299	...

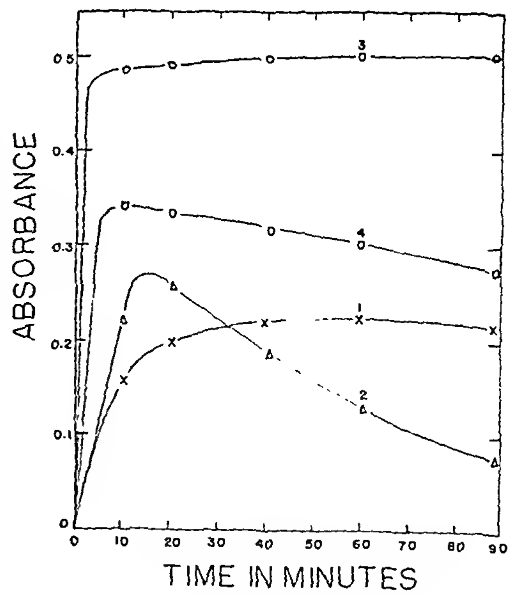

Fig. 2.—Formation and stability of color at 20°. (1) Naringin: concn. 2.08 γ/ml., max. color and stability, sixty min.; (2) Hesperidin: concn. 4.33 γ/ml., max. color and stability, eighteen to twenty min.; (3) Naringenin: concn. 2.08 γ/ml., max. color and stability, sixty min.; and (4) Hesperetin: concn. 2.08 γ/ml., max. color and stability five to six min. Solvent: Acid reagent, 10-mm. Pyrex cells used.

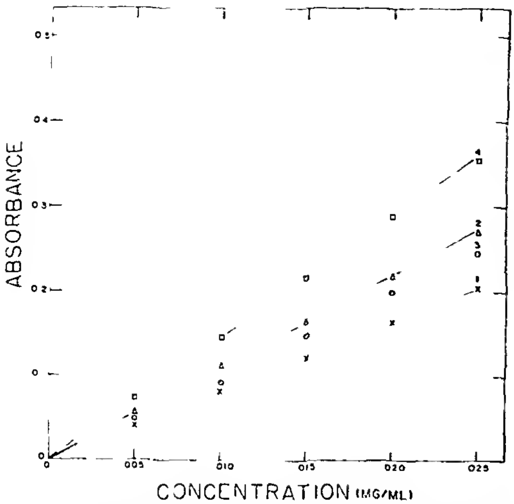

Fig 3.—Adherence to Beer's law. The concentration of hesperidin is four times that indicated, (1) Naringin, (2) Hesperidin, (3) Hesperetin, and (4) Naringenin

four flavanones were not significantly different so they were pooled to give an estimated standard deviation per determination of 0.0014 (absorbance). This does not include weighing errors. This is considerably less than the error for other methods used to determine flavanones quantitatively.

Factors Influencing Color Development.—*Rate of Reduction.*—The best replication was obtained by adding the acetic acid in the following manner: The tube containing the solution to be reduced was placed over a magnetic stirrer and a Teflon-covered stirring bar was inserted. While the solution was being stirred rapidly, the acetic acid was allowed to drop in slowly. In order to control the drop rate a fine capillary was used on the top of the pipet which changed its flow rate to eight minutes, see Fig. 4. After four minutes, at which time most of the acetic acid had drained, the capillary was removed and the last amount in the pipet was allowed to drain normally.

Temperature.—Different temperatures during the color development step greatly influence the rate and intensity of the color development. Figure 5 uses hesperidin as an example to illustrate the time required to attain maximum absorbance at 0, 20, and 40°. As 20° allows ample time before maximum color occurs and yet is not excessive, this temperature was selected. However, lower temperatures may be employed to produce greater stability.

Acid Concentration.—As the ratio of the hydrochloric acid to the acetic acid is increased in the "acid reagent," the color intensity increases. However, a larger proportion of hydrochloric acid causes the color to become less stable. In order to obtain a moderate degree of color stability and yet retain sensitivity, the ratio of hydrochloric acid to acetic acid was empirically chosen. When using the potassium borohydride method qualitatively, the sensitivity can be greatly increased by larger amounts of hydrochloric acid in the "acid reagent."

Stability of Color.—Figure 2 shows the development and decay of color intensity at 20°. Since naringin and naringenin color is quite stable after thirty minutes development, they are read at thirty minutes. Hesperidin and hesperetin are read, preferably, at eighteen to twenty minutes and five to six minutes, respectively.

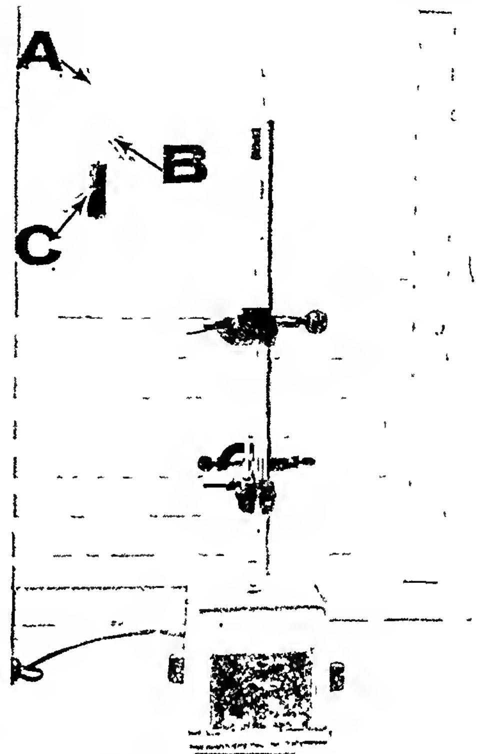

Fig. 4—Apparatus for controlled reduction with acetic acid. A, capillary; B, glass tube with glass wool packing to protect capillary; and C, rubber sleeve for attachment of capillary to pipet.

Stability of Flavanones in Final Dilute Alkaline Solution.—The flavanones will decompose slightly in alkaline solutions when held at 20° for several hours. It is, therefore, necessary that the holding time before reduction be as short as practical. See Table II.

TABLE II.—STABILITY OF FLAVANONES IN ALKALINE SOLUTION

Flavanone	Absorbance Readings 15 min	60 min	300 min
Hesperidin	0.270	0.270	0.259
Hesperetin	0.363	0.360	0.360
Naringin	0.210	0.200	0.195
Naringenin	0.235	0.235	0.230

Each alkaline solution was held at 20° for the designated time and the reduction and color formation steps followed as outlined under procedure. Since the samples used were *not* anhydrous, higher values would have resulted if the samples had been dried and handled carefully so as to avoid moisture absorption.

Absorptivity Values on Anhydrous Flavanones.[1]—

[1] The term anhydrous flavanones used here pertains to materials dried for two hours at 105°.

TABLE III.—ANALYSIS FOR HESPERIDIN COMPLEX IN FINISHED PHARMACEUTICALS

Preparation	Hesperidin Complex[d] added to each tablet, mg	Recovery,[e] %	Calcd Amount Determined, mg
Cold tablet[a]	20	103 6, 99 4, 101 8	20 7, 19.9, 20 9
	10	99 4, 102 4, 100 6	9 9, 10 2, 10.1
Multivitamin[b]	20	99 4, 102 0, 106 0	19 9, 20 4, 21.2
	10	103 0, 104 0, 104 8	10 3, 10 4, 10.5
Liver and iron[c]	20	104.3, 104 2, 105 3	20 9, 20 8, 21 1
	10	103 2, 103 0, 97 4	10 3, 10 3, 9 7

[a] Contains thonzylamine HCl, aspirin, phenacetin, caffeine, and ascorbic acid
[b] Commercial multivitamin and mineral preparation
[c] Commercial liver and iron preparation containing, in addition, copper, B_{12}, and ascorbic acid
[d] Orange Products Division, Sunkist Growers, Ontario, Calif
[e] Absorbance of test/absorbance of control × 100 = per cent recovery

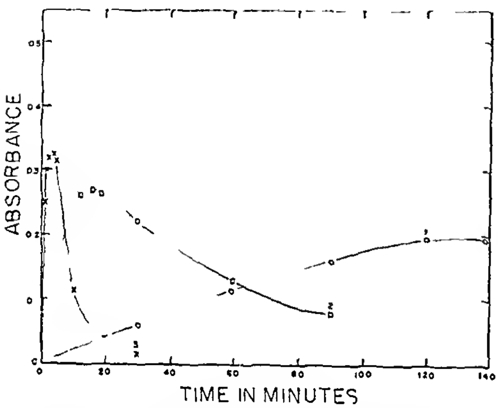

Fig 5.—The intensity and stability of the color developed by reduced hesperidin at different temperatures: (1) 0°, (2) 20°, and (3) 40°.

The following list represents the absorptivity values obtained on the purified materials used in this study: hesperidin, (a) = 34,000, hesperetin, (a) = 181,000, naringin·$2H_2O$, (a) = 114,000, naringenin, (a) = 250,000.

More effective methods of purification may result in higher values However, the authors have been unable to obtain materials from any source with absorptivity values higher than these

Application of the Method to Finished Pharmaceuticals.—Many preparations containing hesperidin plus ascorbic acid and excipients may be analyzed as under "Analytical Procedure" Complex preparations may be successfully analyzed by removing the water solubles before analysis The following technique has proved to be adequate with all preparations tested to date

Procedure.—Grind the finished pharmaceutical to pass through a 100-mesh screen Weigh sufficient material to contain 20 mg of the hesperidin product used in the preparation Weigh 20 mg from the lot of hesperidin used to serve as a control Place each sample in a 50-ml centrifuge tube and add 20 ml of water which was previously saturated with hesperidin to which a small amount of antifoam (1 mg / 100 ml Dow-Corning AF) has been added Mix and centrifuge at 2,000 r p m for five minutes Examine centrifuged tubes for floating particles—especially in the control, and recentrifuge if necessary Decant carefully and repeat washing a second time Decant and solubilize the hesperidin by adding 10 ml of 0 1 *N* NaOH and transfer, quantitatively, to a 25-ml volumetric flask Dilute to volume and filter Dilute the filtrate 1 10 Reduce this dilution and proceed as under "Analytical Procedure" The absorbance value of the test sample is compared to that of the control and the per cent recovery calculated

Table III illustrates the precision obtained from three complex preparations The results are of triplicate analyses When the pharmaceuticals themselves, without hesperidin, were tested none showed even a trace of flavanone content

SUMMARY

The potassium borohydride method for flavanones offers a rapid, simple, quantitative means of determining these compounds and has the following distinct advantages over previous methods: (*a*) Only with flavanones are red to violet colors produced, (*b*) Amounts as low as 5 mcg per ml can be determined quantitatively (Hesperidin 20 mcg/ml), and (*c*) The reduction may be conducted without external cooling For the past two years this method has been used in connection with other methods to determine the flavanone content in hesperidin, naringin, naringenin, and hesperetin products Consistent results have been obtained and considerable time saved in analysis The method has been shown to be adaptable to hesperidin analyses in complex pharmaceutical preparations

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The Synthesis of Some Derivatives of Cinnamic Acid and Their Antifungal Action*

By H. WAYNE SCHULTZ† and GAIL A. WIESE

Various amide derivatives of *o*-chlorocinnamic acid, *p*-nitrocinnamic acid, and cinnamic acid were prepared and tested for antifungal activity against four organisms. N-(4-Chlorophenyl)-2-chlorocinnamamide proved to be the most active compound against *M. gypseum* and *T. rubrum*, while N-(3-chlorophenyl) cinnamamide proved to be the most active compound against *M. canis* and *T. mentagrophytes*. In general, the results of the test indicated that most of the cinnamamide derivatives had little or no antifungal activity *in vitro*.

Aromatic compounds represent a class of antifungal agents that have been successfully used in treating superficial mycoses. In reviewing the literature concerning these compounds it was noted there have been few investigations directed towards the study of antifungal activity of cinnamic acid derivatives. It was thought that these derivatives might possess antifungal activity because:

(*a*) Cinnamic acid and its derivatives have been reported to have antibacterial, antitubercular, and antifungal activity. In regards to antifungal activity, Uppal (1) found cinnamic acid to be more fungicidal than benzoic acid, while having approximately the same activity as salicylic acid. Also, *o*-chlorocinnamic acid and *p*-nitrocinnamic acid have been reported to have antifungal activity (2).

(*b*) The relationship of cinnamic acid to fungistatic benzoic acid derivatives. Benzoic acid, of which cinnamic acid is a vinylog, and its derivatives have received considerable investigation as antifungal agents. One of these derivatives, 2-*n*-amyloxybenzamide, has been found to be more effective than many of the commonly used antifungal agents. Also, various salicylic acid derivatives, such as salicylanilide (3) and the phenylsalicylamides (4) have been reported to have therapeutic value as antifungal agents.

(*c*) The presence of alpha-beta unsaturation in the molecule. Several investigators have reported increased antifungal activity in compounds due to the presence of alpha-beta unsaturation. Of further significance, cinnamic acid represents the beta-phenyl derivative of acrylic acid, and various derivatives of this acid have been found to have antifungal activity.

Since amide derivatives of other related acids, such as salicylic acid and acrylic acid, have shown useful antifungal properties, the cinnamamide derivatives were selected to be prepared and tested. Furthermore, a review of the literature revealed that amides of cinnamic acid have not been investigated for antifungal activity.

EXPERIMENTAL PART I

The following processes exemplify the various methods used for the preparation of the cinnamic acid derivatives. The *o*-chlorocinnamoyl chloride was prepared from *o*-chlorocinnamic acid[1] by the method as recorded by Andrews, Van Campen, and Schulman (5). Information concerning the various cinnamamides which were prepared is summarized in Table I.

Method I.—N - (2 - Chlorophenyl) - 2 - chlorocinnamamide.—In a 500-ml. round-bottom flask, equipped with a reflux condenser, was placed 10 Gm. (0.05 mole) of *o*-chlorocinnamoyl chloride. A solution of 6.4 Gm. (0.05 mole) *o*-chloroaniline, dissolved in 30 ml. of C. P. grade pyridine, was added slowly to this. The reaction mixture was refluxed for three hours and then poured slowly, with stirring, into a beaker containing 300 ml. of distilled water. The water solution was cooled and the crystalline material removed with suction filtration and dried. The product was purified by recrystallization from ethyl alcohol. The yield was 7.9 Gm. (54.1% of theoretical) of white crystals which melted at 170.5 to 171.5°.

Method II.—N - (2 - Chlorophenyl) - 4 - nitrocinnamamide.—In a 500-ml. round-bottom flask was placed 20 Gm. (0.1 mole) of *p*-nitrocinnamic acid and 50 ml. benzene. The mixture was cooled in an ice bath and 19.2 Gm. (0.16 mole) of thionyl chloride, dissolved in 15 ml. benzene, was added, dropwise. The reaction mixture was refluxed for one and one-fourth hours, after which time complete solution was effected. The excess thionyl chloride was removed by distillation under reduced pressure. Two fresh portions of 50 ml. benzene were added and distilled from the acid chloride to facilitate removal of the thionyl chloride. To the resulting acid chloride, dissolved in 75 ml. benzene, a solution of 26.5 Gm. (0.21 mole) of *o*-chloroaniline in 50 ml. benzene was added, dropwise. The reaction mixture was refluxed for one-half hour, after which the solvent was removed by evaporation. The resulting product was recrystallized from methyl ethyl ketone. The yield

* Received June 20, 1959, from the State University of Iowa College of Pharmacy, Iowa City.
Abstracted from a dissertation submitted to the Graduate College of the State University of Iowa by H. Wayne Schultz in partial fulfillment of the requirements for the degree of Doctor of Philosophy, February 1959.
† Present address: School of Pharmacy, Oregon State College, Corvallis.

[1] The *o*-chlorocinnamic acid used in this study was supplied through the courtesy of the Hayden-Newport Chemical Corp., New York, N. Y.

TABLE I.

Compound	Method	Yield, %	M. p., ° C.	Recrystn. Solvent	N, % Calcd.	N, % Found
N-(2-Chlorophenyl) cinnamamide	I	45.8	137–138	EtOH	5.44	5.39
N-(3-Chlorophenyl) cinnamamide	I	27.9	125–126	EtOH	5.44	5.62
N-(4-Chlorophenyl) cinnamamide	I	59.4	185–186	EtOH	5.44	5.40
N-(2,4-Dichlorophenyl) cinnamamide	I	35.8	162–164	EtOH	4.79	4.89
N-(2,5-Dichlorophenyl) cinnamamide	I	22.2	177–178	EtOH	4.79	4 83
N-(2-Benzothiazyl) cinnamamide	I	47.4	245–246	EtOH-acetone	10.00	9.84
N-(3-Quinolyl) cinnamamide	I	61.0	217–218	EtOH	10.21	10.17
N-(5-Chloro-2-pyridyl) cinnamamide	I	59.2	186–187	EtOH	10.82	10.63
N-(2-Methyl-4-chlorophenyl) cinnamamide	I	29.4	189–190	EtOH	5.16	4.96
N-(2-Chlorophenyl)-2-chlorocinnamamide	I	54.1	170.5–171.5	EtOH	4.80	4.70
N-(3-Chlorophenyl)-2-chlorocinnamamide	I	77.4	162–163	Benzene	4.80	4.71
N-(4-Chlorophenyl)-2-chlorocinnamamide	I	76.7	190–190.5	Chloroform	4.80	4.74
N-(2,4-Dichlorophenyl)-2-chlorocinnamamide	I	69.2	177–178	CCl_4	4.29	4.21
N-(2,5-Dichlorophenyl)-2-chlorocinnamamide	I	76.0	158–159	CCl_4	4.29	4.19
N-(2-Benzothiazyl)-2-chlorocinnamamide	I	F6.8	240–241	Me cellosolve	8.91	8.71
N-(3-Quinolyl)-2-chlorocinnamamide	I	62.2	213–214.5	EtOH	9.08	8.87
N-(5-Chloro-2-pyridyl)-2-chlorocinnamamide	I	54.6	231–232	EtOH	9.56	9.39
N-(2-Methyl-4-chlorophenyl)-2-chlorocinnamamide	I	54.5	291–292	EtOH	4.58	4.76
N-(2-Chlorophenyl)-4-nitrocinnamamide	II	66.1	220–221	Me Et ketone	9.26	8.99
N-(3-Chlorophenyl)-4-nitrocinnamamide	III	68.9	196–197.5	EtOH	9.26	8.94
N-(4-Chlorophenyl)-4-nitrocinnamamide	III	60.4	226–227	EtOH-acetone	9 26	8.96
N-(2,4-Dichlorophenyl)-4-nitrocinnamamide	III	63.3	226–227	EtOH	8.31	8.12
N-(2,5-Dichlorophenyl)-4-nitrocinnamamide	III	59.0	251–253	Pyridine	8.31	8.06
N-(2-Benzothiazyl)-4-nitrocinnamamide	III	47.6	320–321	Pyridine	12.63	12.64
N-(3-Quinolyl)-4-nitrocinnamamide	III	51.6	313–314	Pyridine	13.17	12.89
N-(5-Chloro-2-pyridyl)-4-nitrocinnamamide	III	43.8	262–264	Pyridine	13.85	13.56
N-(2-Methyl-4-chlorophenyl)-4-nitrocinnamamide	III	49.7	260–261	Pyridine-EtOH	8.85	8.55

was 20.0 Gm (66.1% of theoretical), of light yellow crystals which melted at 220 to 221°.

Method III.—N - (4 - Chlorophenyl) - 4 - nitrocinnamamide.—In a 500-ml. round-bottom flask was placed 16.1 Gm. (0.083 mole) of *p*-nitrocinnamic acid and 50 ml. benzene. The mixture was cooled in an ice bath and 10.9 Gm. (0.09 mole) of thionyl chloride, dissolved in 15 ml. benzene was added dropwise. The reaction mixture was refluxed for three hours, after which time complete solution was effected. The excess thionyl chloride was removed by distillation under reduced pressure. In order to facilitate the removal of thionyl chloride, 25 ml. toluene was added and distilled from the acid chloride. This purification process was repeated using 25 ml. benzene. To the resulting acid chloride, dissolved in 50 ml. benzene, a solution of 11.6 Gm. (0.09 mole) *p*-chloroaniline in 25 ml. benzene and 25 ml. ether was added, dropwise. The reaction mixture was refluxed for one hour, after which the solvent was removed by evaporation. The product was purified by grinding it into a fine powder, and washing it with two 100-ml. 2% hydrochloric acid, two 100-ml. water, two 100-ml. 2% sodium carbonate, and two 100-ml. water. The resulting product was recrystallized from equal parts of ethyl alcohol and acetone. The yield was 16.2 Gm. (60.4% of theoretical) of light yellow crystals, which melted at 226 to 227°.

EXPERIMENTAL PART II

The compounds were tested for antifungal activity using a modified paper disk technique similar to that described by Kligman and Rosenweig (6). The test organisms used were: *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, and *Trichophyton mentogrophytes*. Acetone solutions of the compounds were prepared in concentrations of 4, 2, 1, and 0.5%. Saturated solutions were prepared for compounds whose solubility in acetone were less than 0.5%. Two known antifungal agents, undecylenic acid and salicylanilide, were tested as reference compounds.

Sterile filter paper disks (12.7 mm. in diameter) were immersed for thirty seconds in each of the acetone solutions of the compounds. The excess liquid was drained off by touching the disk to the wall of the container, and the disk was placed immediately on the agar surface of a Petri dish which had been previously inoculated with the desired test organism. On each Petri dish were placed two filter disks saturated with the acetone solution. After incubation for fifteen days at room temperature, the zones of inhibition, representing the minimum distance between the periphery of the filter disk and the colony growth, were recorded as the mean of the two measurements. Only those compounds which had a zone of inhibition greater than 3 mm. are given in Table II.

DISCUSSION

In the separation and purification of the *p*-nitrocinnamic acid derivatives, it was necessary to wash the crude product with a dilute hydrochloric acid solution first, in order to remove the excess amine, and then to wash the remaining product well with dilute sodium hydroxide solution, to remove the excess *p*-nitrocinnamic acid. This procedure was

TABLE II.—MINIMUM WIDTH[a] OF ZONES OF INHIBITION, IN MILLIMETERS

Compound	w/v, %	*M canis*	*M gypseum*	*T rubrum*	*T mentagrophytes*
N-(2-Chlorophenyl) cinnamamide	0.5	0	0.5	0	0
	1.0	0	1	0	0
	2.0	0	2	0	0
	4.0	1	3.5	1	0
N-(3-Chlorophenyl) cinnamamide	0.5	1	5	1	0
	1.0	2.5	6.5	1	0.5
	2.0	4.8	7	1	9
	4.0	7	8	1	11.5
N-(3-Chlorophenyl)-2-chlorocinnamamide	0.5	1	2	0	1
	1.0	1.5	3	1	1.5
	2.0	3.5	3.5	3	2.5
	4.0	5	5	4	4.5
N-(4-Chlorophenyl)-2-chlorocinnamamide	0.5	0	6.5	0.5	0
	1.0	0	8.5	1.5	0
	2.0	0	10	2.5	0
	4.0	1.5	24	10	0
N-(2,5-Dichlorophenyl)-2-chlorocinnamamide	0.5	0	1.5	0	0
	1.0	2	2	0	0
	2.0	3	3	0	0
	4.0	...[b]	...[b]	...[b]	...[b]
Salicylanilide	0.5	9	9.5	7.5	6
	1.0	14	10.5	9	14
	2.0	20	14	10	18
	4.0	24	19	12.5	22.5
Undecylenic acid	0.5	4	10.5	7	5
	1.0	6.5	11.5	11	11
	2.0	10	16	14.5	16
	4.0	14	25	20	20
Acetone	0.5	0	0	0	0
	1.0	0	0	0	0
	2.0	0	0	0	0
	4.0	0	0	0	0

[a] Average of two measurements [b] Insoluble in acetone at the stated concentration

necessitated due to the low solubility of the amides in organic solvents, as well as the high degree of insolubility of *p* nitrocinnamic acid (a contaminant) in the solvents

It was desired in this investigation to test and compare the compounds at various concentrations to determine the effect of concentration on antifungal properties Because of the insoluble nature of most of the compounds, particularly the *o*-chloro and *p*-nitro derivatives, it was not possible to test all of the compounds at several different concentrations However, it was decided to prepare and test saturated solutions of these "insoluble" compounds to determine if any of these compounds would exert antifungal activity when at a concentration which was equal to their maximum solubility, i e , a saturated solution

The results of the test indicated that most of the cinnamamide derivatives had little or no antifungal activity *in vitro* N(4-Chlorophenyl)-2-chlorocinnamamide proved to be the most active of the compounds tested against *M. gypseum*, having activity greater than salicylanilide while being comparable to undecylenic acid at 4% concentration. Also, it was the most active of the compounds against *T rubrum*, having activity less than salicylanilide and much less than undecylenic acid. Against *M. canis* and *T mentagrophytes*, N-(3-chlorophenyl) cinnamamide proved to be the most active compound In general, it appeared that substitution on either the phenyl ring of cinnamic acid or variation of the amine portion of the molecule had little effect on antifungal activity of these compounds

The apparent lack of antifungal activity as shown by the tested compounds may be due to several factors Perhaps, chief among these factors would be the general insolubility (both in water and organic solvents) that was exhibited by these compounds. Also, it may be possible that the testing procedure disguised the true antifungal activity of these compounds Since the zones of inhibition are greatly dependent upon the ability of compounds to diffuse in the agar, the diffusability factor, rather than the inhibitory factor may have been measured Although the testing method used represents a useful screening procedure for antifungal activity, it may not correlate the *in vitro* results to clinical effectiveness

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N-(3-Chlorophenyl) cinnamamide	0.5	1	5	1	0
	1.0	2.5	6.5	1	0.5
	2.0	4.8	7	1	9
	4.0	7	8	1	11.5
N-(3-Chlorophenyl)-2-chlorocinnamamide	0.5	1	2	0	1
	1.0	1.5	3	1	1.5
	2.0	3.5	3.5	3	2.5
	4.0	5	5	4	4.5
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	1.0	0	8.5	1.5	0
	2.0	0	10	2.5	0
	4.0	1.5	24	10	0
N-(2,5-Dichlorophenyl)-2-chlorocinnamamide	0.5	0	1.5	0	0
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Acetone	0.5	0	0	0	0
	1.0	0	0	0	0
	2.0	0	0	0	0
	4.0	0	0	0	0

[a] Average of two measurements [b] Insoluble in acetone at the stated concentration

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